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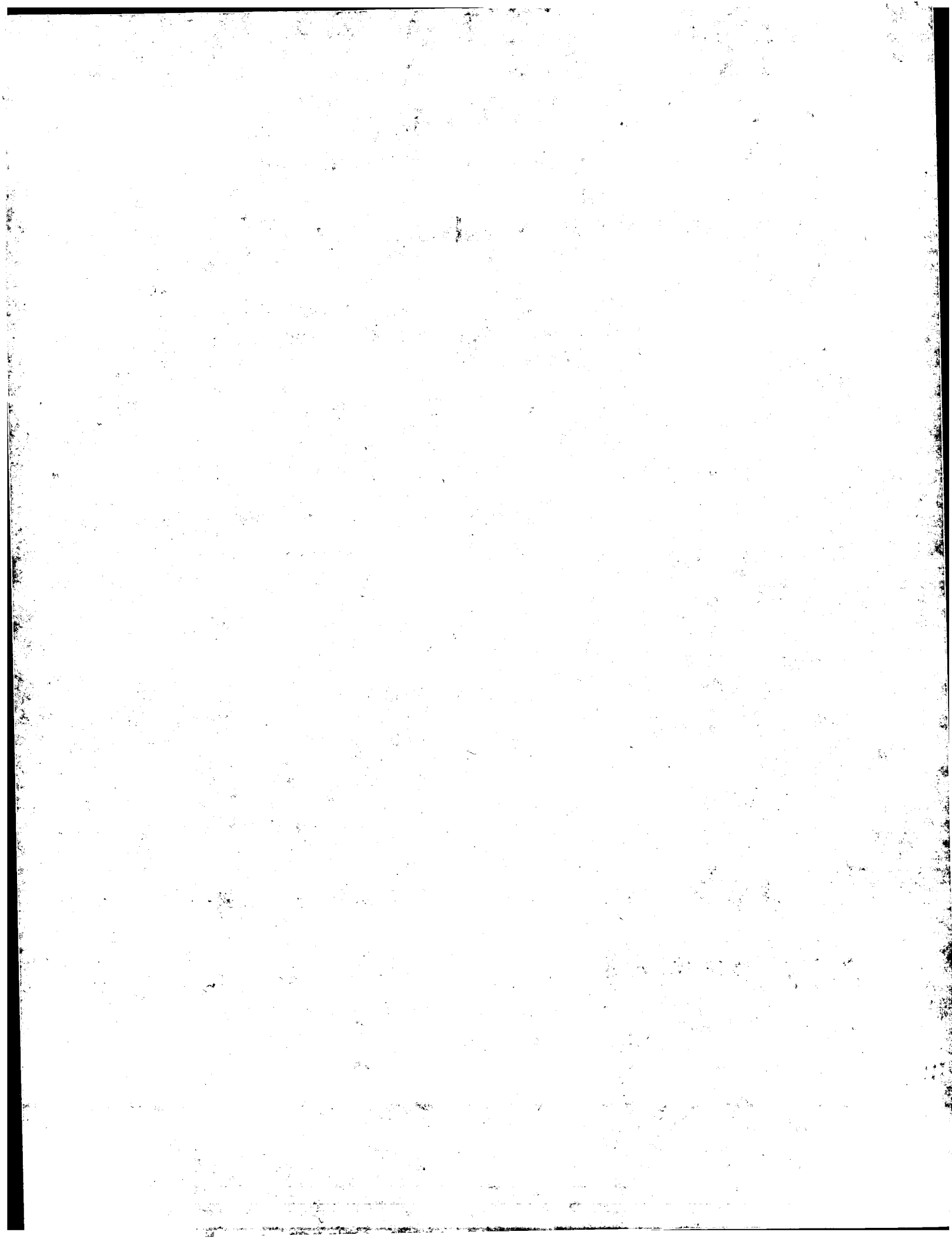
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(54) Title: USE OF PROTEGRINS FOR PERIODONTAL INDICATIONS		
(57) Abstract <p>The invention is directed to a method to treat periodontal disease which method comprises administering to a subject afflicted with such disease an amount of a protegrin effective to treat said disease; wherein said protegrin contains the amino acid sequence (1): A₁-A₂-A₃-A₄-A₅-C₆-A₇-C₈-A₉-A₁₀-A₁₁-A₁₂-C₁₃-A₁₄-C₁₅-A₁₆-A₁₇-A₁₈, wherein said protegrin contains 10-30 amino acid residues, wherein the amino acid sequence of formula (1) may be extended at the N and/or C-terminus by additional noninterfering amino acids; and the N-terminal acylated and/or C-terminal amidated or esterified forms thereof, said protegrin either in the optionally -SH stabilized linear or in a disulfide-bridged form wherein each of C₆, C₈, C₁₃ and C₁₅ is independently a cysteine, homocysteine, or penicillamine, or wherein one or more of C₆, C₈, C₁₃ and C₁₅ is independently replaced by a basic, hydrophobic, large/polar or small amino acid or wherein C₈ and/or C₁₃ is not present; each of A₁-A₅ is independently present or not present, and if present each is independently a basic, hydrophobic, polar/large, or small amino acid; each of A₇ and A₁₄ is independently a hydrophobic or a small amino acid; A₉-A₁₂ are capable of effecting a β-turn when contained in the compound of formula (1) and at least one of A₉-A₁₂ must be a basic amino acid and wherein A₉ and/or A₁₂ may be present or not present; each of A₁₆-A₁₈ is independently present or not present, and if each present each is independently a basic, hydrophobic, polar/large or small amino acid; wherein in said protegrin at least about 15 % to about 50 % of the amino acids are basic amino acids, and wherein the protegrin compound has a net positive charge of at least +1 at physiological pH.</p>		

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USE OF PROTEGRINS FOR PERIODONTAL INDICATIONS

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5 No. A122839 from NIH-NIAID. The U.S. Government has certain rights in this invention.

Technical Field

The invention relates to the field of treating
10 periodontal pathogens. In particular, the invention concerns the use of protegrins in the context of such conditions.

Background Art

15 One of the defense mechanisms against infection by both animals and plants is the production of peptides that have antimicrobial and antiviral activity. Various classes of these peptides have been isolated from tissues of both plants and animals. PCT application WO 95/03325
20 published 2 February 1995 contains a review of the literature on this subject. Such peptides include tachyplesins, which are 17-18 amino acid peptides containing four invariant cysteines, the defensins, β -defensins, and insect defensins, which are somewhat
25 longer peptides characterized by six invariant cysteines and antifungal and antibacterial peptides and proteins which have been found in plants.

The antimicrobial peptides used in the present invention are members of a class designated "protegrins".
30 Representative members of protegrins have been isolated from porcine leukocytes; however, the isolated peptides are simply exemplary members of a class of peptides that are effective against periodontal disease.

The isolation of certain protegrin peptides and
35 related peptides or of DNA encoding them has been

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reported by Kokryakov, V.N. et al. *FEBS Lett* (1993) 337:231-236; Zhao, C. et al., *FEBS Lett* (1994) 346:285-288; Mirgorodskaya, O.A. et al. *FEBS Lett* (1993) 330:339-342; Storici, P. et al. *Biochem Biophys Res Comm* (1993) 196:1363-1367; Harwig, S.S.L., et al. *J Peptide Sci* (1995) 3:207; Zhao, C., et al. *FEBS Lett* (1995) 376:130-134; Zhao, C. et al. *FEBS Lett* (1995) 368:197-202; Miyakawa, Y. et al. *Infect Immun* (1996) 64:926-932; Yasin, B. et al. *Infect Immun* (1996) 64:709-713; Qu, X-D et al. *Infect Immun* (1996) 64:1240-1245; Aumelas, A. et al. *Eur J. Biochem* (1996) 237:575-583; Mangoni, M.E. et al. *FEBS Lett* (1996) 383:93-98; and a paper from the Eighth International Symposium on Staph Infections, Aix les Bains, France, June 23-26, 1996, Steinberg, D.A. et al., entitled "Protegrins: Fast Acting Bactericidal Peptides." The general protegrin group of peptides is also disclosed in U.S. Serial No. 08/499,523 and in U.S. Serial No. 08/690,921, the contents of which are incorporated herein by reference in their entirety.

20 The peptide class designated "protegrins" has a wide range of antimicrobial activity and varies in activity spectrum by member of class.

The protegrins have been found to bind to endotoxins -- i.e., the lipopolysaccharide (LPS) compositions derived from Gram-negative bacteria which are believed responsible for Gram-negative sepsis. The protegrins are also effective in inhibiting the growth of organisms that are associated with sexually transmitted diseases such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae*.

30 Protegrins are also effective against the microorganisms associated with oral mucositis, a significant side effect of cancer therapy and bone marrow transplantation that is not adequately managed by current

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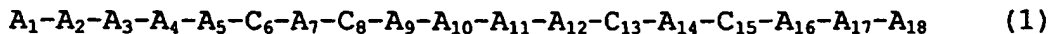
approaches (Sonis, S.T. In: J.L. Holland et al. Cancer Medicine, 3rd ed. Lea and Febiger, Philadelphia (1993a) pp. 2381-2388; Sonis, S.T. In: V. DeVitta et al. (ed.), Principles and Practice of Oncology. J.B. Lippincott, Philadelphia (1993b) pp. 2385-2394).

The present invention is directed to the use of the protegrins to treat periodontal conditions. Specifically, the protegrins have been found effective in bactericidal or bacteriastatic activity against Gram negative, facultative periodontal pathogens.

Disclosure of the Invention

The present invention is directed to treatment periodontal disease. Specifically, the protegrins alone or in combination are effective in ameliorating the symptomology caused by infection with *Actinobacillus actinomycetemcomitans* and *Capnocytophaga* Spp.

Accordingly, in one aspect, the invention is directed to a method to treat periodontal conditions which method comprises administering to a subject afflicted with periodontal disease an amount of a compound that includes the amino acid sequence of formula 1, alone or in combination with other medicaments, in an amount effective to ameliorate or otherwise treat said periodontal condition. The protegrins of the invention contain an amino acid sequence of the formula



and contains 10-30 amino acid residues. The sequence shown as (1) can be extended at the N and/or C-terminus with non-interfering amino acids or peptide sequence. Also included are the N-terminal acylated and/or C-terminal amidated or esterified forms thereof,

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and the protegrins may be either in the optionally -SH stabilized linear or in a disulfide-bridged form.

Each of C₆, C₈, C₁₃ and C₁₅ shown in amino acid sequence (1) is independently a cysteine, homocysteine, or penicillamine; or alternatively, one or more of C₆, C₈, C₁₃ and C₁₅ is independently replaced by a basic, hydrophobic, large/polar or small amino acid; or C₈ and/or C₁₃ is not present;

each of A₁-A₅ is independently present or not present, and if present each is independently a basic, hydrophobic, polar/large, or small amino acid;

each of A₇ and A₁₄ is independently a hydrophobic or a small amino acid;

A₉-A₁₂ are capable of effecting a β -turn when contained in the protegrin and at least one of A₉-A₁₂ must be a basic amino acid, optionally A₉ and/or A₁₂ may not be present;

each of A₁₆-A₁₈ is independently present or not present, and if each present each is independently a basic, hydrophobic, polar/large or small amino acid.

In all the compounds containing the sequence of formula (1) at least about 15% and no more than about 50% of the amino acids must be basic amino acids, and the compounds must have a net charge of at least +1 at physiological pH. The invention is also related to periodontal compositions containing the protegrins.

Brief Description of the Drawings

Figure 1 shows the structures of PG-1, PG -2, PG-3, PG-4 and PG-5.

Figure 2A, 2B and 2C are graphic representations of the activity of various concentrations of PG-1 versus Gram-negative bacteria important in periodontal disease.

Figure 3A-3F are graphic representations of the activity of various protegrins against Gram-negative bacteria causative of periodontal disease.

Figures 4A-4C are graphical representations of the effect of serum on the bactericidal activity of PG-1 against periodontal microorganisms.

Figure 5 is the graphic representation of bactericidal activity of PG-1 as affected by salt concentration.

10

Modes of Carrying Out the Invention

The protegrins useful in the invention include the amino acid sequence:

15 $A_1-A_2-A_3-A_4-A_5-C_6-A_7-C_8-A_9-A_{10}-A_{11}-A_{12}-C_{13}-A_{14}-C_{15}-A_{16}-A_{17}-A_{18}$ (1)

and its defined modified forms. The designation A_n in each case represents an amino acid at the specified position in the peptide. As defined, A_1-A_5 , C_8 , A_9 , A_{12} ,

20 C_{13} , A_{16} , A_{17} and/or A_{18} may or may not be present.

However, the peptides of the invention contain 10-30 amino acids. Thus, the amino acid sequence shown as formula (1) may contain extensions at the N and/or C-terminus of additional amino acids or peptide sequence.

25 The positions of the cysteine, homocysteine or penicillamine residues, shown as C in the formula, are invariant in one embodiment of the peptides of the invention; however, in the modified forms, also included within the scope of the invention, one or more of these
30 cysteine, homo-cysteine or penicillamines may be replaced by a small, basic or hydrophobic amino acid. All of the protegrins useful in the invention, however, have a net positive charge; approximately 15% but no more than about 50% of the amino acids must be basic amino acids, and the
35 compounds must have a net charge of at least +1 at

physiological pH. For embodiments having as few as 10 amino acids, there may be only one basic amino acid residue; however, at least two basic residues, even in this short-chain residue, are preferred. If the
5 protegrin contains as many as 15 amino acid residues, two basic residues are required. It is preferred that at least 20% of the amino acids in the sequence be basic, more preferably 30%.

The active protegrins are also preferably contain a
10 β turn bracketed by two strands that form a β sheet. While not intending to be bound by any theory, applicants believe that antimicrobial activity of the compounds of formula (1) is associated with such a β -turn bracketed by two strands that form a β sheet structure. The amino
15 acids A_9 - A_{12} must be capable of effecting a β -turn, which can be encouraged by hydrogen bonding between A_9 and A_{12} . The presence of proline at A_{10} and/or A_{11} does not interfere with the β -turn stabilized by the presence of a hydrophobic amino acid at positions A_9 or A_{12} .

20 As used herein, " β -turn" refers to a recognized subclass of reverse-turns. Typically, a " β -turn" is a four amino acid residue peptide segment that reverses the direction of a polypeptide chain so as to allow a single polypeptide chain to adopt an anti-parallel β -sheet
25 secondary structure. Generally, the two internal amino acid residues of the β -turn are not involved in the hydrogen-bonding of the β -sheet; the two amino acid residues on either side of the internal residues are included in the hydrogen-bonding of the β -sheet. The
30 term " β -turn" expressly includes all types of peptide β -turns commonly known in the art including, but not limited to, type-I, type-II, type-III, type-I', type-II', and type-III' β -turns (see, Rose et al., 1985, *Adv. Protein Chem.* 37:1-109; Wilmer-White et al., 1987, *Trends*

Biochem. Sci. **12**:189-192; Wilmot et al., 1988, *J. Mol. Biol.* **206**:759-777; Tramontano et al., 1989, *Proteins; Struct. Funct. Genet.* **6**:382-394).

The presence of the four invariant cysteine, homo-
5 cysteine or penicillamine residues is helpful in
effecting the β -sheet structure; however, by properly
choosing the substitutions, one or more of the cysteine,
homocysteine or penicillamine residues can be replaced
without substantially disturbing the three-dimensional
10 shape of the molecule.

The β sheets are believed to be effected by C₆-A₇-C₈
and C₁₃-A₁₄-C₁₅. Thus, in the unmodified forms of the
compound, A₇ and A₁₄ are preferably hydrophobic amino
acids. The cysteine, homocysteine or penicillamine
15 residues may also, then, be replaced by other residues
which do not affect the maintenance of the β sheet
formation; these substitutions would include basic,
hydrophobic or small amino acids.

The amino terminus of the protegrin may be in the
20 free amino form or may be acylated by a group of the
formula RCO-, wherein R represents a hydrocarbyl group of
1-25C, preferably 1-10C, more preferably 1-8C. The
hydrocarbyl group is saturated or unsaturated, straight
chain or cyclic, and is typically, for example, methyl,
25 ethyl, i-propyl, t-butyl, n-pentyl, cyclohexyl,
cyclohexene-2-yl, hexene-3-yl, hexyne-4-yl, octyl, decyl,
eicososyl and the like.

The C-terminus of the protegrin may be in the form
of the underivatized carboxyl group, either as the free
30 acid or an acceptable salt, such as the potassium,
sodium, calcium, magnesium, or other salt of an inorganic
ion or of an organic ion such as caffeine. In some
embodiments, it is difficult to make salts since the
remainder of the molecule bears a positive charge which

may repel the relevant cation. The carboxyl terminus may also be derivatized by formation of an ester with an alcohol of the formula ROH, or may be amidated by an amine of the formula NH_3 , or RNH_2 , or R_2NH , wherein each R is independently hydrocarbyl of 1-25C as defined and with preferred embodiments as above. Amidated forms of the peptides wherein the C-terminus has the formula CONH_2 are preferred.

Addition of lipophilic groups at the C- and/or N-terminus facilitates the transition of the peptide into the membrane of the target microbe. Choice of optimum substitution is determined by evaluation with respect to the lipid content of the target microbe.

As the protegrins contain substantial numbers of basic amino acids, the peptides of the invention may be supplied in the form of the acid addition salts. Typical acid addition salts include those of inorganic ions such as chloride, bromide, iodide, fluoride or the like, sulfate, nitrate, or phosphate, or may be salts of organic anions such as acetate, formate, benzoate and the like. The acceptability of each of such salts is dependent on the intended use, as is commonly understood.

The protegrins that contain at least two cysteine, homocysteine or penicillamines may be in straight-chain or cyclic form, due to disulfide bond formation. The cyclic forms are the result of the formation of disulfide linkages among all or some of the four invariant cysteine, homocysteine or penicillamine residues. Cyclic forms of the invention include all possible permutations of disulfide bond formation. The straight-chain forms are convertible to the cyclic forms, and vice versa. Methods for forming disulfide bonds to create the cyclic peptides are well known in the art, as are methods to reduce disulfides to form the linear compounds. The

linear compounds can be stabilized by addition of a suitable alkylating agent such as iodoacetamide.

The native forms of the protegrins contain two disulfide bonds are between the cysteine, homocysteine or penicillamine at position 6 and the cysteine, homocysteine or penicillamine at position 15 and the other between the cysteine, homocysteine or penicillamine at position 8 and the cysteine, homocysteine or penicillamine at position 13. Accordingly, in those embodiments having two disulfide linkages, the C₆-C₁₅, C₈-C₁₃ form is preferred. However, forms of the protegrins containing only one disulfide linkage are active and easily prepared. Preferred among embodiments having only one disulfide linkage are those represented by C₆-C₁₅ alone and by C₈-C₁₃ alone.

Forms containing a C₆-C₁₅ disulfide as the only disulfide linkage are generally designated "bullet" forms of the protegrins; those wherein the sole disulfide is C₈-C₁₃ are designated the "kite" forms. The bullet and kite forms can most conveniently be made by replacing the cysteine, homocysteine or penicillamines at the positions not to be involved in a disulfide linkage preferably with a small amino acid such as glycine, serine, alanine or threonine. Alternatively, C₈, C₁₃ or both may be absent.

As the linearized or "snake" forms of the native cyclic peptides have valuable activities, even when chemically stabilized to preserve the sulfhydryl form of cysteine, homocysteine or penicillamine for example, by reaction with iodoacetamide, the protegrins also include linearized forms which are stabilized with suitable reagents. As defined herein, "SH-stabilized" forms of the peptides of the invention contain sulfhydryl groups reacted with standard reagents to prevent reformation into disulfide linkages. Alternatively the cysteine, homocysteine or penicillamine residues are replaced by

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small or basic amino acids as set forth above. It is preferred that all 4 cysteine, homocysteine or penicillamine residues be replaced in order to minimize the likelihood of the formation of intermolecular disulfide links.

The amino acids denoted by A_n (and the cysteine, homocysteine or penicillamine residues) may be those encoded by the gene or analogs thereof, and may also be the D-isomers thereof. One preferred embodiment is that form wherein all of the residues are in the D-configuration thus conferring resistance to protease activity while retaining antimicrobial or antiviral properties. The resulting protegrins are themselves enantiomers of the native L-amino acid-containing forms.

In one class of protegrins, either the hydrophobic amino acids found in the native protegrins at A_5 and/or A_{16} are replaced with a basic amino acid and/or at least one of A_1 - A_4 is hydrophobic and/or at least one, and preferably all four of amino acids A_1 and A_4 found in the native forms are deleted; and/or one or more of A_5 , C_8 , A_9 , A_{12} , C_{13} and A_{16} is absent. By suitable manipulation of these and other features, the range of conditions under which the class of protegrins of the present invention are effective can be varied. Furthermore, the spectrum of microbes against which they are effective can also be modified. This is further described hereinbelow.

The amino acid notations used herein are conventional and are as follows:

Amino Acid	One-Letter Symbol	Three-Letter Symbol
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamine	Q	Gln
Glutamic acid	E	Glu
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

The amino acids not encoded genetically are abbreviated as indicated in the discussion below.

5 In the specific peptides shown in the present application, the L-form of any amino acid residue having an optical isomer is intended unless the D-form is expressly indicated by a dagger superscript ([†]).

10 The compounds of the invention are peptides which are partially defined in terms of amino acid residues of designated classes. Amino acid residues can be generally subclassified into major subclasses as follows:

15 **Acidic:** The residue has a negative charge due to loss of H ion at physiological pH and the residue is attracted by aqueous solution so as to seek the surface positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium at physiological pH.

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Basic: The residue has a positive charge due to association with H ion at physiological pH and the residue is attracted by aqueous solution so as to seek the surface positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium at physiological pH.

Hydrophobic: The residues are not charged at physiological pH and the residue is repelled by aqueous solution so as to seek the inner positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium.

Polar/large: The residues are not charged at physiological pH, but the residue is not sufficiently repelled by aqueous solutions so that it would necessarily seek an inner position in the conformation of the peptide in which it is contained when the peptide is in aqueous medium. Depending on the conditions, and on the remaining amino acids in the sequence, the residue may reside either in the inner space or at the surface of the protein.

This description also characterizes certain neutral amino acids as "small" since their side chains are not sufficiently large, even if polar groups are lacking, to confer hydrophobicity. "Small" amino acids are those with four carbons or less when at least one polar group is on the side chain and three carbons or less when not.

It is understood, of course, that in a statistical collection of individual residue molecules some molecules will be charged, and some not, and there will be an attraction for or repulsion from an aqueous medium to a greater or lesser extent. To fit the definition of "charged," a significant percentage (at least approximately 25%) of the individual molecules are charged at physiological pH. The degree of attraction or repulsion required for classification as polar or

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nonpolar is arbitrary and, therefore, amino acids specifically contemplated by the invention have been classified as one or the other. Most amino acids not specifically named can be classified on the basis of known behavior.

Amino acid residues can be further subclassified as cyclic or noncyclic, and aromatic or nonaromatic, self-explanatory classifications with respect to the side-chain substituent groups of the residues, and as small or large. The residue is considered small if it contains a total of four carbon atoms or less, inclusive of the carboxyl carbon, provided an additional polar substituent is present; three or less if not. Small residues are, of course, always nonaromatic.

For the naturally occurring protein amino acids, subclassification according to the foregoing scheme is as follows.

Acidic	Aspartic acid and Glutamic acid
Basic	Noncyclic: Arginine, Lysine Cyclic: Histidine
Small	Glycine, Serine, Alanine, Threonine
Polar/large	Asparagine, Glutamine
Hydrophobic	Tyrosine, Valine, Isoleucine, Leucine, Methionine, Phenylalanine, Tryptophan

The gene-encoded secondary amino acid proline is a special case due to its known effects on the secondary conformation of peptide chains, and is not, therefore, included in a group. Cysteine, homocysteine or penicillamine residues are also not included in these classifications since their capacity to form disulfide bonds to provide secondary structure is critical in the compounds of the present invention.

Certain commonly encountered amino acids, which are not encoded by the genetic code, include, for example,

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β -Alanine (β -Ala), or other omega-amino acids, such as 3-aminopropionic, 2,3-diaminopropionic (2,3-diaP), 4-aminobutyric and so forth, α -aminisobutyric acid (Aib), sarcosine (Sar), ornithine (Orn), citrulline (Cit),
5 t-butylalanine (t-BuA), t-butylglycine (t-BuG), N-methylisoleucine (N-MeIle), phenylglycine (Phg), and cyclohexylalanine (Cha), norleucine (Nle), 2-naphthylalanine (2-Nal); 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic); β -2-
10 thienylalanine (Thi); methionine sulfoxide (MSO); and homoarginine (Har). These also fall conveniently into particular categories.

Based on the above definitions,

Sar, β -Ala, and Aib are small;

15 t-BuA, t-BuG, N-MeIle, Nle, Mvl, Cha, Phg, Nal, Thi and Tic are hydrophobic;

Orn, 2,3-diaP and Har are basic;

Cit, Acetyl Lys, and MSO are polar/large.

The various omega-amino acids are classified
20 according to size as small (β -Ala and 3-aminopropionic) or as large and hydrophobic (all others).

Other amino acid substitutions of those encoded in the gene can also be included in peptide compounds within the scope of the invention and can be classified within
25 this general scheme according to their structure.

In all of the peptides of the invention, one or more amide linkages (-CO-NH-) may optionally be replaced with another linkage which is an isostere such as -CH₂NH-, -CH₂S-, -CH₂CH₂-, -CH=CH- (cis and trans), -COCH₂-,
30 -CH(OH)CH₂- and -CH₂SO-. This replacement can be made by methods known in the art. The following references describe preparation of peptide analogs which include these alternative-linking moieties: Spatola, A.F., Vega Data (March 1983), Vol. 1, Issue 3, "Peptide Backbone

Modifications" (general review); Spatola, A.F., in Chemistry and Biochemistry of Amino Acids Peptides and Proteins, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983) (general review); Morley, J.S., *Trends Pharm Sci* (1980) pp. 463-468 (general review); Hudson, D., et al., *Int J Pept Prot Res* (1979) **14**:177-185 (-CH₂NH-, -CH₂CH₂-); Spatola, A.F., et al., *Life Sci* (1986) **38**:1243-1249 (-CH₂-S); Hann, M.M., *J Chem Soc Perkin Trans I* (1982) 307-314 (-CH-CH-, cis and trans); Almquist, R.G., et al., *J Med Chem* (1980) **23**:1392-1398 (-COCH₂-); Jennings-White, C., et al., *Tetrahedron Lett* (1982) **23**:2533 (-COCH₂-); Szelke, M., et al., European Application EP 45665 (1982) CA:97:39405 (1982) (-CH(OH)CH₂-); Holladay, M.W., et al., *Tetrahedron Lett* (1983) **24**:4401-4404 (-C(OH)CH₂-); and Hruby, V.J., *Life Sci* (1982) **UB**:189-199 (-CH₂-S-).

In addition to analogs which contain isosteres in place of peptide linkages, the peptides or proteins of the invention include peptide mimetics in general, such as those described by Olson, G.L. et al. *J Med Chem* (1993) **36**:3039-3049 and retro-inverso type peptides as described by Chorev, M. et al. *Science* (1979) **204**:1210-1212; and Pallai, P.V. et al., *Int J Pept Protein Res* (1983) **21**:84-92.

The compounds of formula (1) are generally defined as set forth in the Disclosure of the Invention set forth above.

In preferred embodiments, all of the cysteine, homocysteine or penicillamines at positions 6, 8, 13 and 15 are present as are A₉ and A₁₂.

In addition, or alternatively, each of A₇ and A₁₄ is a hydrophobic amino acid, preferably Ile, Val, Leu, Trp, Phe, or Tyr. In another set of preferred embodiments, all of A₁-A₄ are not present or at least one, and

- 16 -

preferably two of A₁-A₄ is a hydrophobic amino acid, preferably Ile, Val, Leu, Trp, Phe or Tyr.

In another set of preferred embodiments, A₉-A₁₂ contain at least one hydrophobic amino acid residue, preferably Phe, Tyr or Trp.

Other preferred embodiments include those wherein each of A₁ and A₉ is independently selected from the group consisting of R, K and Har; more preferably, both A₁ and A₉ are R; however, each of A₁ and A₉ may be absent.

In another class of preferred embodiments, each of A₂ and A₃ is independently selected from the group consisting of G, A, S and T, or I, V, L, F, Y, or W; more preferably, A₂ and A₃ are G, W, F, Y, L, or V; however, A₂ and/or A₃ may be absent.

In another set of preferred embodiments, A₄ is selected from the group consisting of R, K, H, Orn, Har, G, A, S, T, F, Y and W; more preferably, A₄ is R, G or W; however, A₄ may be absent.

In another set of preferred embodiments, each of A₅ and A₁₆ is independently selected from the group consisting of I, V, L, Nle, W, Y, and F, preferably I, V, L, W, F and Y. However, A₅ and/or A₁₆ may be absent.

In another set of preferred embodiments, each of A₇ and A₁₄ is independently selected from the group consisting of I, V, L, W, Y and F, preferably A₇ is I, F, Y or W and A₁₄ is I, V, L, W, Y, or F.

In another set of preferred embodiments, one of A₉ and A₁₂ is R, K, H, Orn or Har, preferably R and the other is I, V, L, Nle, W, Y or F, preferably R, F or W.

In another set of preferred embodiments, A₁₀ is R, G, W or P.

In another set of preferred embodiments, A₁₁ is R, G, W or P.

A₁₇ is preferably absent, but when present, is preferably G, A, S or T;

- 17 -

A₁₈ is preferably absent, but when present, is preferably R, K, H, Orn or Har, most preferably R.

Also preferable when all four amino acids A₁-A₄ are present, preferably A₁ and A₄ are basic and A₂ and A₃ are small amino acids, and at least one of A₁-A₄ is a small or hydrophobic amino acid. Preferred embodiments of A₁-A₄ include R-G-G-R, R-G-W-R, R-L-L-R and the like.

As described above, the compounds of formula (1) are either in disulfide or noncyclic (linearalized) form or may be modified wherein one or more cysteine, homocysteine or penicillamines is replaced by a small amino acid residue, a basic amino acid residue or a hydrophobic amino acid residue. If the linearalized forms of the compound of formula (1) are prepared, or if linearalized forms of those modified peptides which contain at least two cysteine, homocysteine or penicillamines are prepared, it is preferred that the sulfhydryl groups be stabilized by addition of a suitable reagent. Preferred embodiments for the basic amino acid to replace cysteine, homocysteine or penicillamine residues are R, K, H and Har, preferably R or K. Preferred small amino acids to replace the cysteine, homocysteine or penicillamine residues include G, A, S and T, most preferably A and T.

The compounds of the invention may thus contain either two disulfide bonds, one disulfide bond, or no disulfide bonds. Where two disulfide bonds are present, as described above, those corresponding to the disulfide bonds in naturally occurring protegrins are preferred -- i.e., C₆-C₁₅ and C₈-C₁₃. Where only one disulfide bond is present, it is preferred that the protegrin be in the bullet or kite form. This can be assured by replacing at least one noninvolved cysteine, homocysteine or penicillamine with an alternative amino acid as described above.

The parent applications herein describe members of the protegrin family which are isolated from porcine leukocytes. Five such protegrins have been found, PG-1 through PG-5 with the following amino acid sequences:

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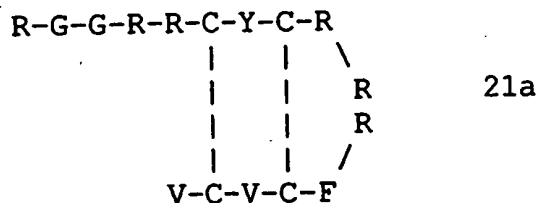
Unmodified forms

PG-1: R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V-G-R
 PG-2: R-G-G-R-L-C-Y-C-R-R-R-F-C-I-C-V
 PG-3: R-G-G-G-L-C-Y-C-R-R-R-F-C-V-C-V-G-R
 10 PG-4: R-G-G-R-L-C-Y-C-R-G-W-I-C-F-C-V-G-R
 PG-5: R-G-G-R-L-C-Y-C-R-P-R-F-C-V-C-V-G-R

In the form present in the porcine leukocytes, it is believed that the C-terminal amino acids are amidated and
 15 that there are two disulfide linkages as described above. However, linearalized forms of these native protegrins are also biologically active. Particularly preferred compounds of the invention are those which are similar to the native forms but are in the enantiomeric form-i.e.
 20 all of the amino acids are in this D-configuration. Thus, particularly preferred forms of the unmodified protegrins include the following and their D-enantiomers:

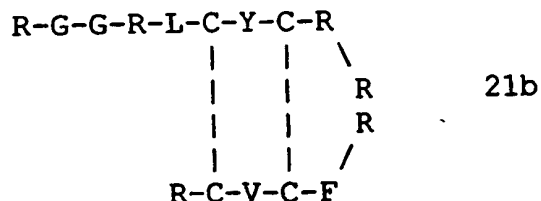
Protegrin form-21

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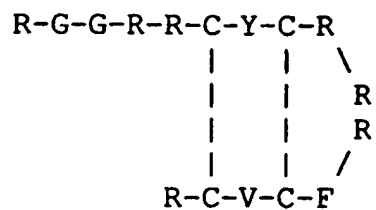
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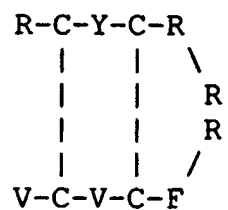


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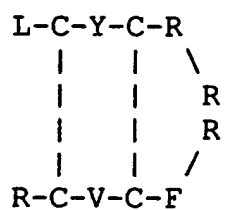
- 19 -



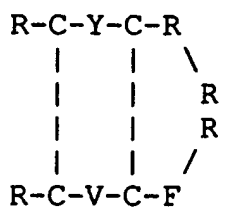
21d



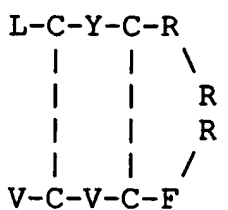
21e



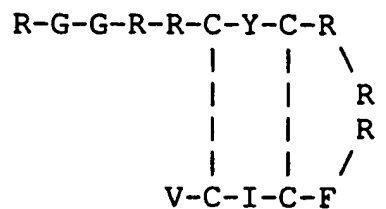
21f



21g



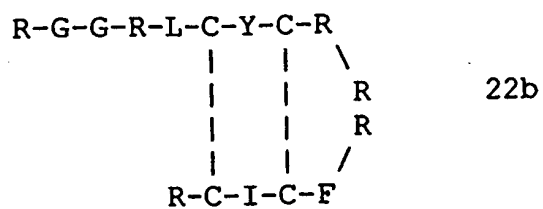
21h

Protegrin form-22

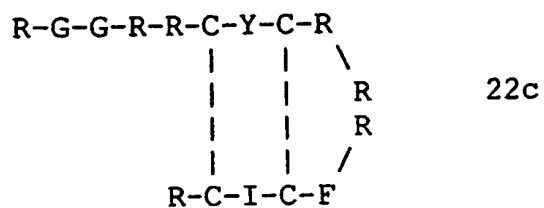
22a

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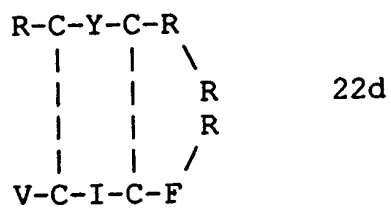


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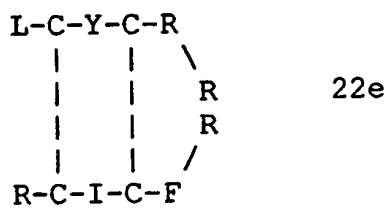
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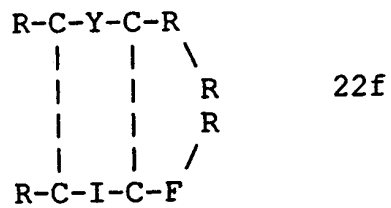


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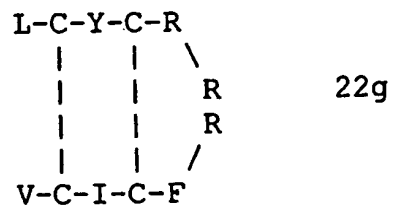


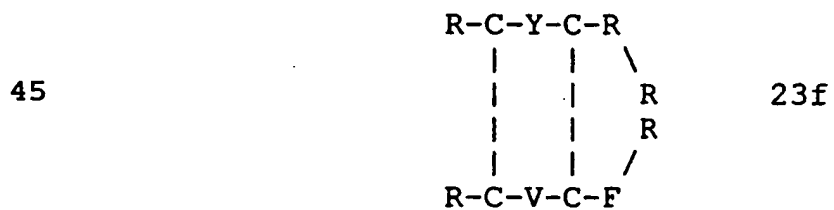
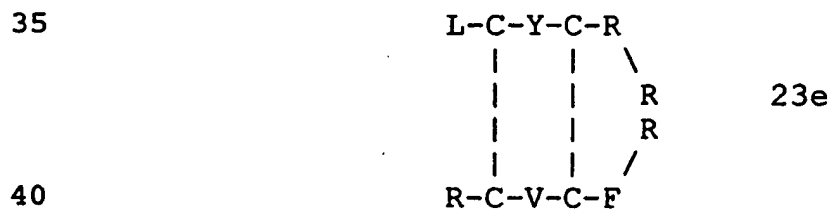
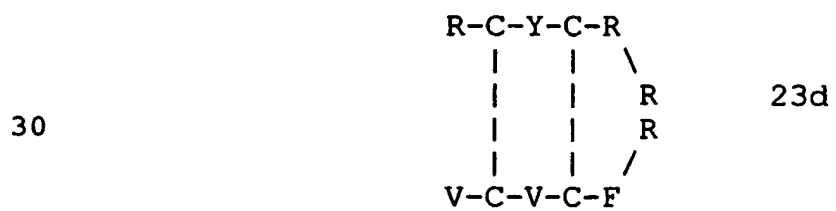
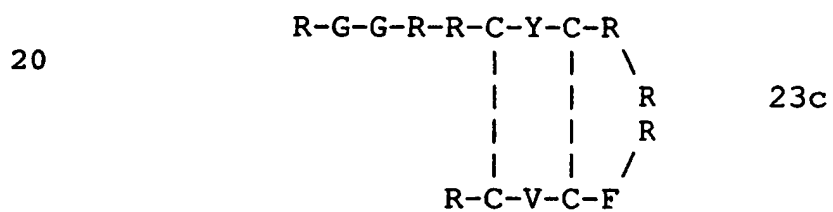
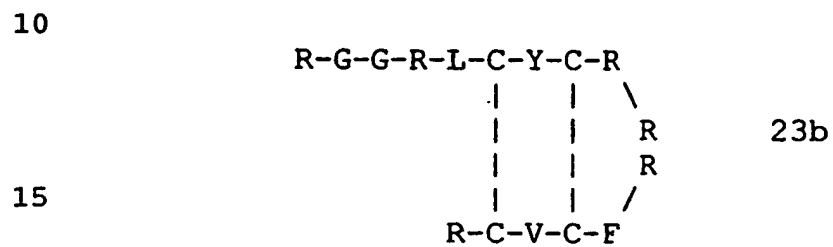
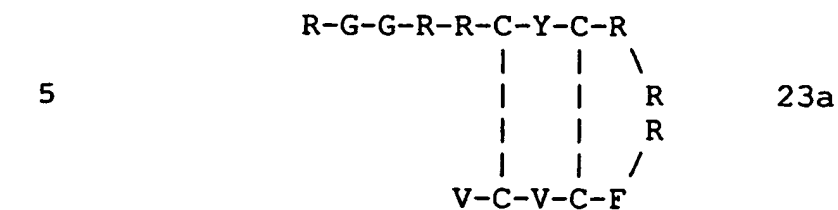
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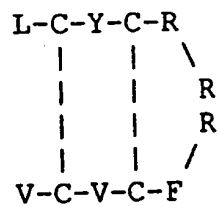
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Protegrin form-23

- 22 -

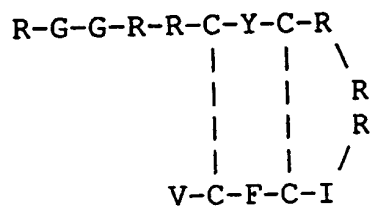
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23g

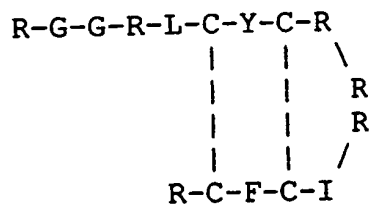
Protegrin form-24

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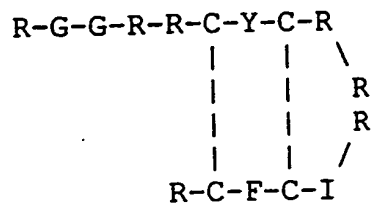
24a

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24b

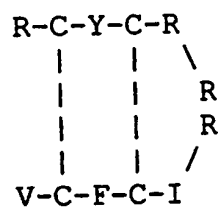
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24c

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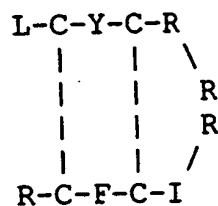
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24d

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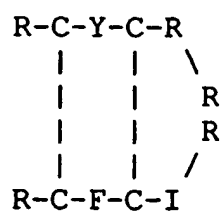


24e

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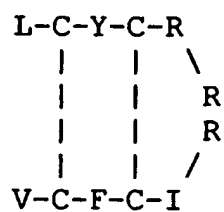
- 23 -

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24f

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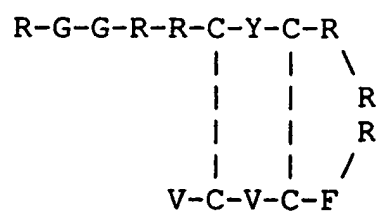


24g

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Protegrin form-25

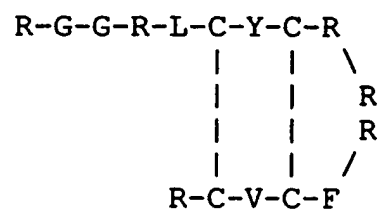
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25a

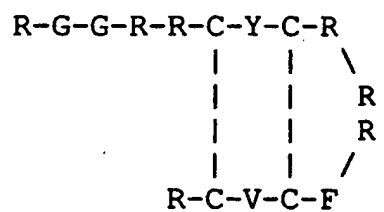
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25b

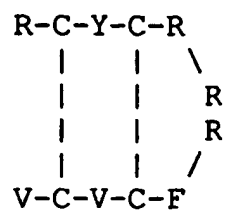
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25c

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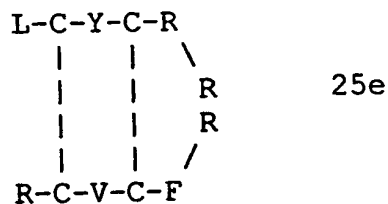


25d

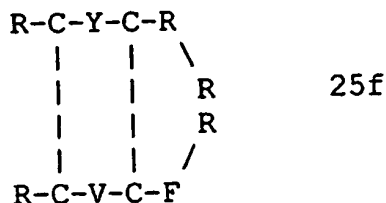
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- 24 -

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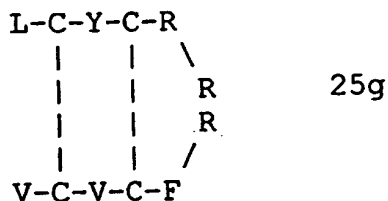


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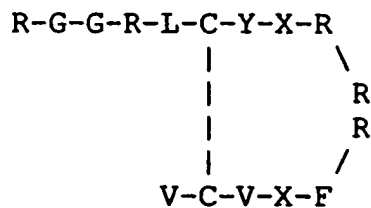


and the foregoing protegrins wherein A_7 is W and/or A_{12} is W and/or wherein A_{14} is W and/or wherein A_{16} is W and/or wherein A_{17} is G and A_{18} is R; and/or wherein at least one of A_5 , A_9 , A_{12} and A_{16} is not present; including the linearalized forms thereof and the N-terminal acylated and C-terminal amidated forms thereof. In the terminology set forth above, protegrin form 21 consists of compounds which are characteristic of the present class but which are otherwise similar to PG-1; forms labeled 22 contain the characteristics of the present class but are modeled after PG-2; classes 23-25 are similarly related to PG-3, PG-4 and PG-5.

Similarly, those compounds of the invention which contain one disulfide bond are preferably selected from the group consisting of the following, including their enantiomeric forms:

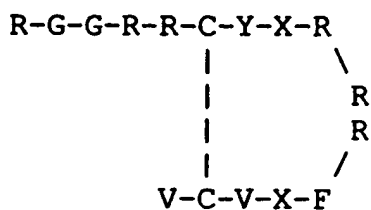
Bullet-21

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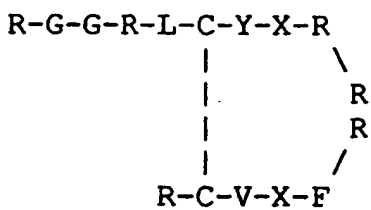
21a

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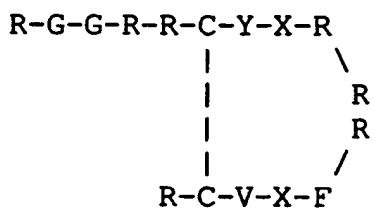
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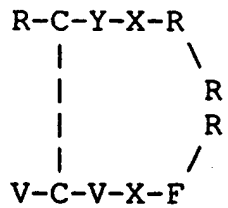
21c

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21d

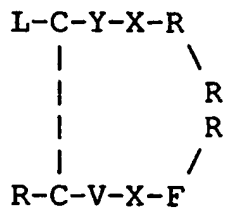
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21e

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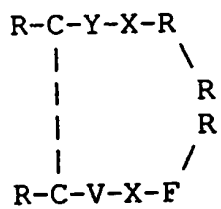
21f

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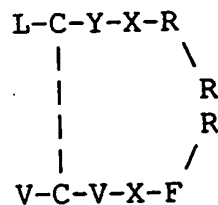
- 26 -

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21g

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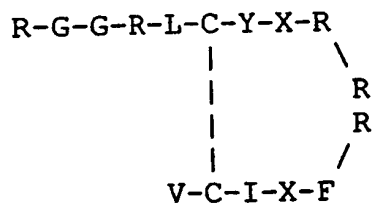


21h

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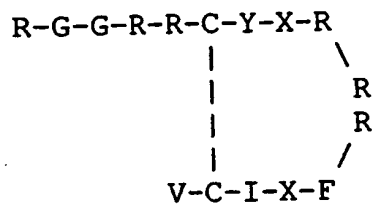
Bullet 22

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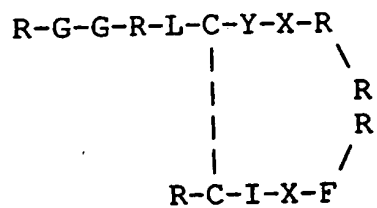
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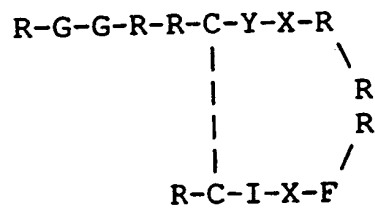
22b

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22c

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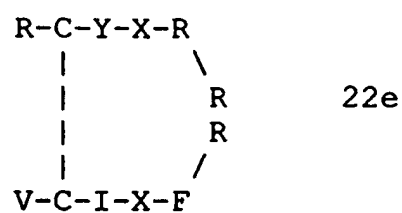
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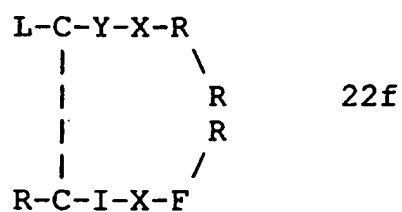
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- 27 -

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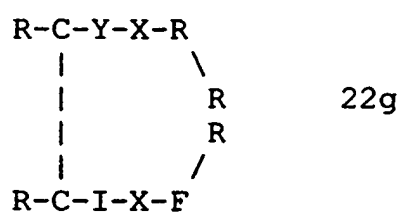


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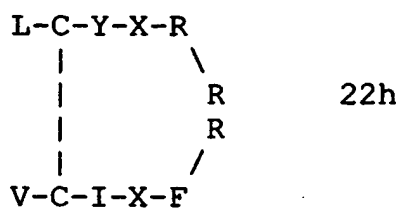


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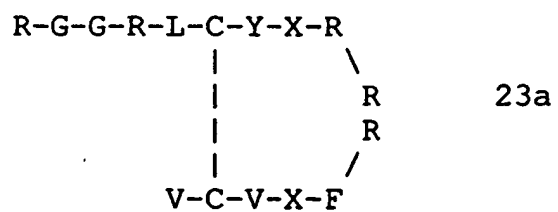
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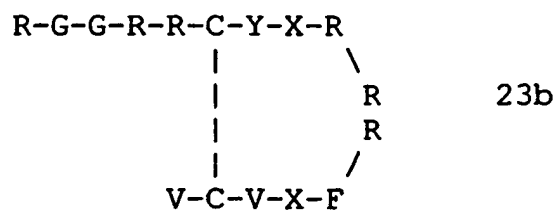
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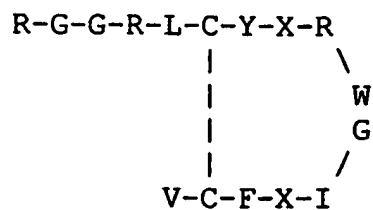


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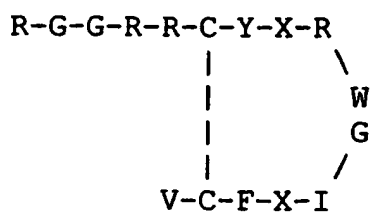
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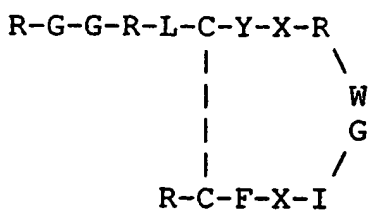
24a

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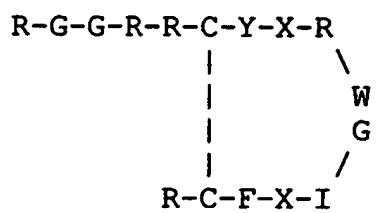
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24c

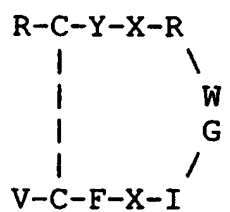
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24d

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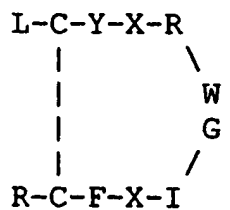
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24e

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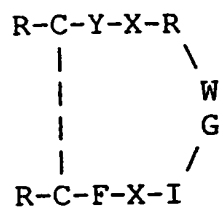


24f

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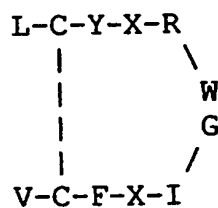
- 30 -

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24g

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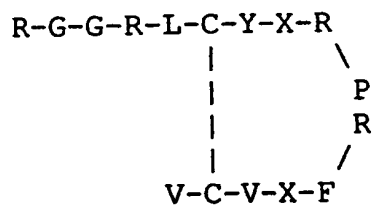


24h

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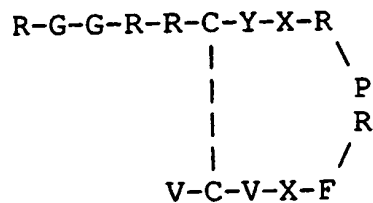
Bullet 25

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25a

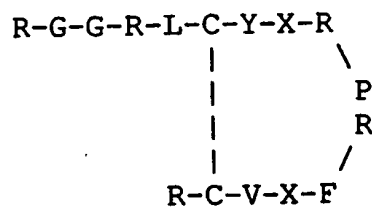
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25b

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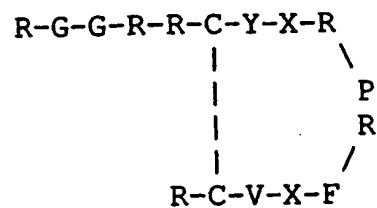
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25c

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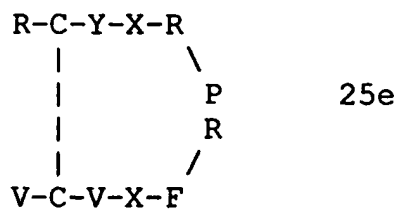


25d

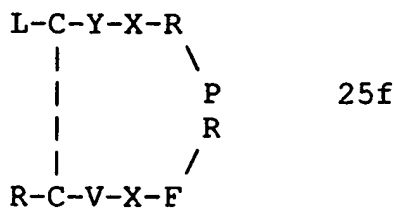
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- 31 -

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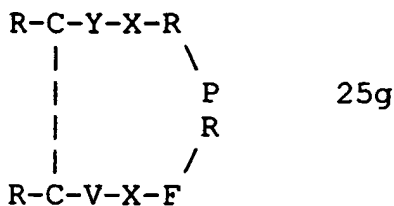


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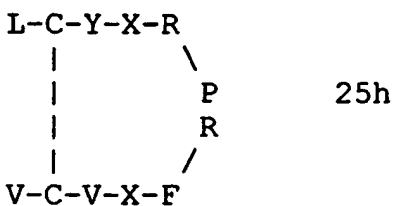


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and the foregoing bullet forms wherein A_7 is W and/or A_{12} is W and/or wherein A_{14} is W and/or wherein A_{16} is W and/or wherein A_{17} is G and A_{18} is R; and/or wherein at least one of A_5 , A_9 , A_{12} and A_{16} is not present,

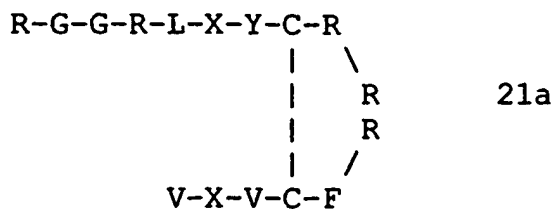
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and the amidated forms thereof,

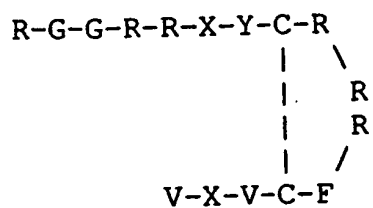
wherein each X is independently a hydrophobic, a small, or a large polar amino acid residue; and the following kite forms, including their enantiomers:

40 Kite form-21

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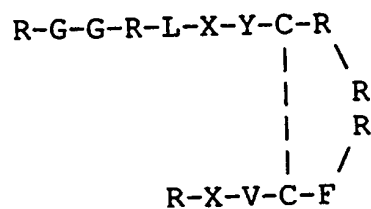


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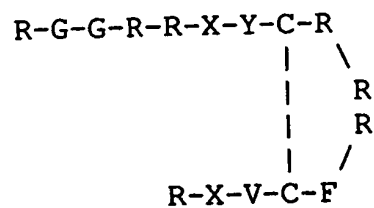
21b

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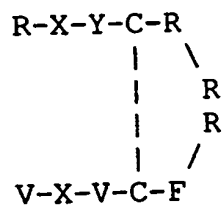
21c

15



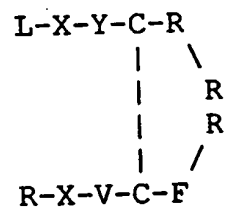
21d

25



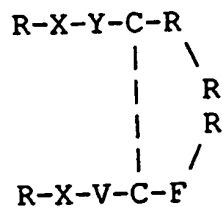
21e

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21f

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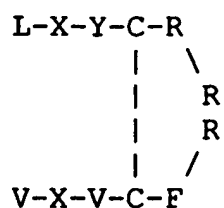
21g

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- 33 -

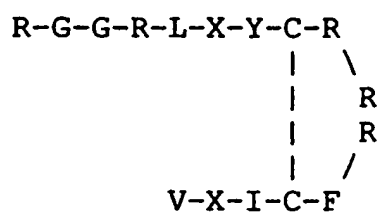
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21h

Kite form-22

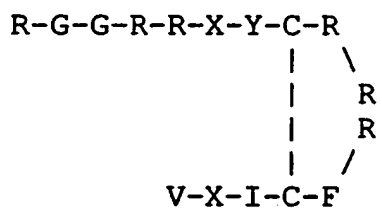
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22a

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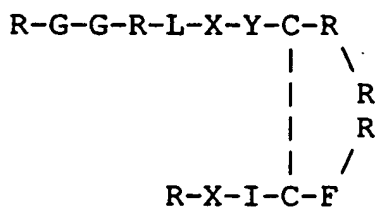
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22b

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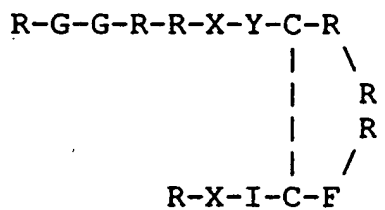
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22c

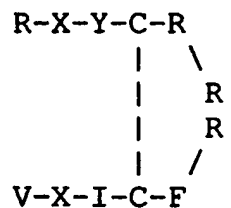
35

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22d

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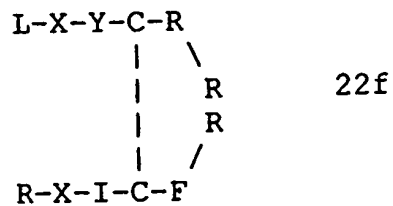


22e

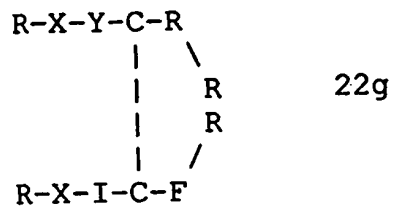
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- 34 -

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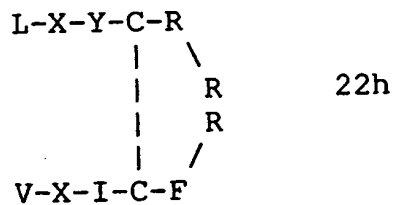


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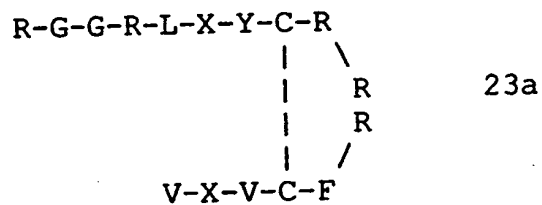


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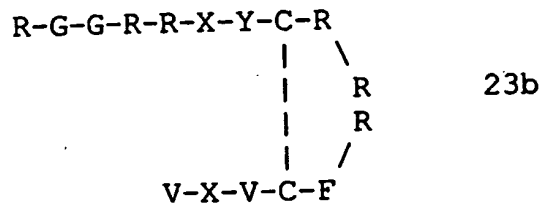
25 Kite form-23

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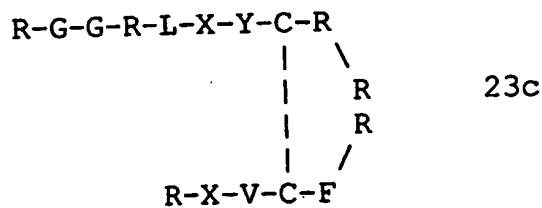


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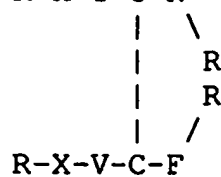


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- 35 -

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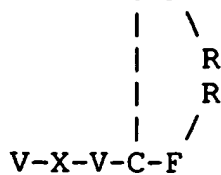
R-G-G-R-R-X-Y-C-R



23d

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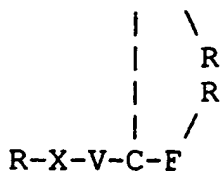
R-X-Y-C-R



23e

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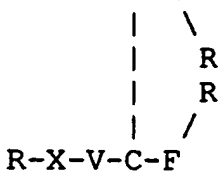
L-X-Y-C-R



23f

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R-X-Y-C-R

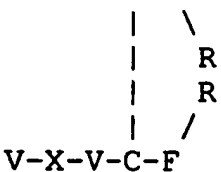


23g

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L-X-Y-C-R



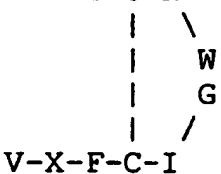
23h

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Kite form-24

R-G-G-R-L-X-Y-C-R



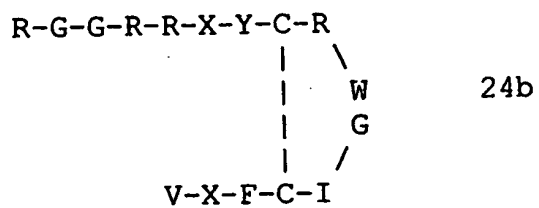
24a

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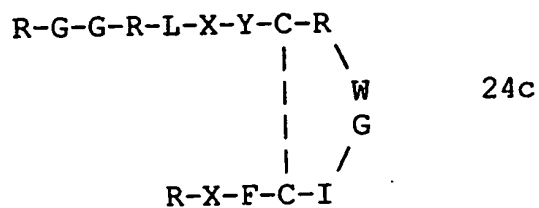
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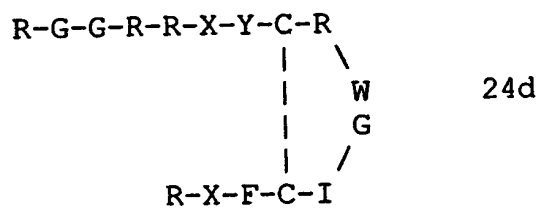


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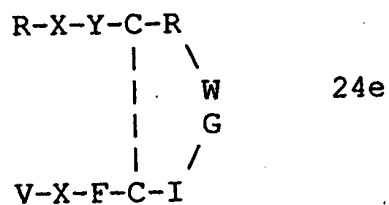
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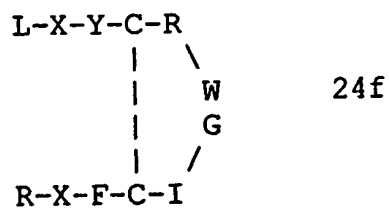


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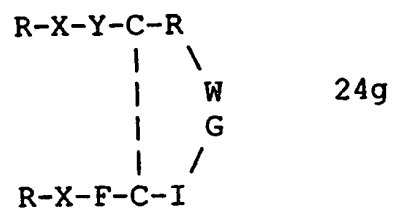


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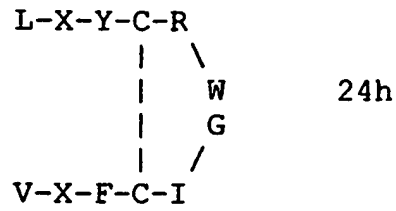
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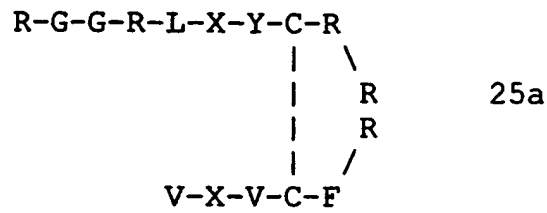


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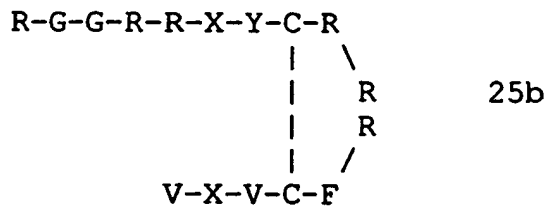
Kite form-25

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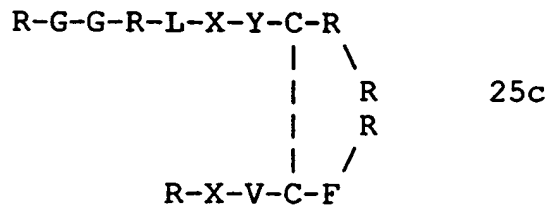
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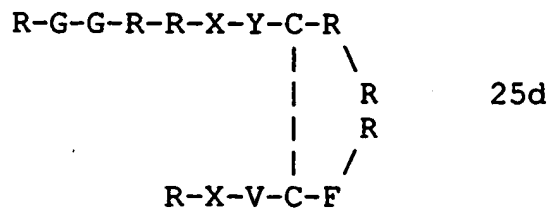


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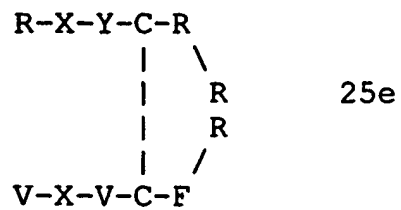


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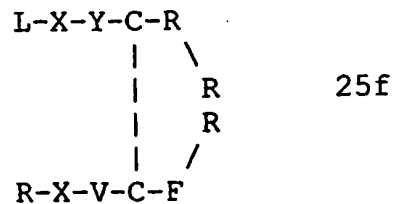
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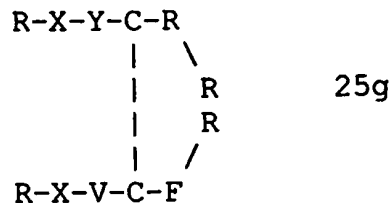
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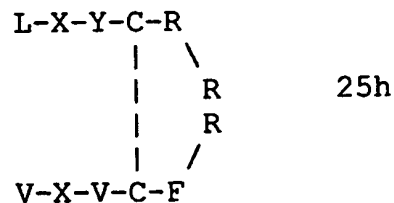


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and the foregoing kite forms wherein A_7 is W and/or A_{12} is W and/or wherein A_{14} is W and/or wherein A_{16} is W and/or wherein A_{17} is G and A_{18} is R; and/or wherein at least one of A_5 , A_9 , A_{12} and A_{16} is not present, and the amidated forms thereof, wherein each X is independently a hydrophobic, small, or large polar amino acid.

These preferred forms also include the linearalized forms, as well as the N-acylated and C-amidated forms. The designation "X" refers to the replacement amino acid as described herein, preferably X is A, S, T or G, most preferably A or T.

Preferred forms which are completely modified by replacement of all cysteine, homocysteine or penicillamine residues are selected from the group consisting of

- 39 -

Snake form-21 R-G-G-R-L-X-Y-X-R-R-R-F-X-V-X-V
 R-G-G-R-R-X-Y-X-R-R-R-F-X-V-X-V
 R-G-G-R-L-X-Y-X-R-R-R-F-X-V-X-R
 R-G-G-R-R-X-Y-X-R-R-R-F-X-V-X-R
 5 R-X-Y-X-R-R-R-F-X-V-X-V
 L-X-Y-X-R-R-R-F-X-V-X-R
 R-X-Y-X-R-R-R-F-X-V-X-R
 L-X-Y-X-R-R-R-F-X-V-X-V

10 Snake form-22 R-G-G-R-L-X-Y-X-R-R-R-F-X-I-X-V
 R-G-G-R-R-X-Y-X-R-R-R-F-X-I-X-V
 R-G-G-R-L-X-Y-X-R-R-R-F-X-I-X-R
 R-G-G-R-R-X-Y-X-R-R-R-F-X-I-X-R
 R-X-Y-X-R-R-R-F-X-I-X-V
 15 L-X-Y-X-R-R-R-F-X-I-X-R
 R-X-Y-X-R-R-R-F-X-I-X-R
 L-X-Y-X-R-R-R-F-X-I-X-V

Snake form-23 R-G-G-G-L-X-Y-X-R-R-R-F-X-V-X-V
 20 R-G-G-G-R-X-Y-X-R-R-R-F-X-V-X-V
 R-G-G-G-L-X-Y-X-R-R-R-F-X-V-X-R
 R-G-G-G-R-X-Y-X-R-R-R-F-X-V-X-R
 R-X-Y-X-R-R-R-F-X-V-X-V
 L-X-Y-X-R-R-R-F-X-V-X-R
 25 R-X-Y-X-R-R-R-F-X-V-X-R
 L-X-Y-X-R-R-R-F-X-V-X-V

Snake form-24 R-G-G-R-L-X-Y-X-R-G-W-I-X-F-X-V
 R-G-G-R-R-X-Y-X-R-G-W-I-X-F-X-V
 30 R-G-G-R-L-X-Y-X-R-G-W-I-X-F-X-R
 R-G-G-R-R-X-Y-X-R-G-W-I-X-F-X-R
 R-X-Y-X-R-G-W-I-X-F-X-V
 L-X-Y-X-R-G-W-I-X-F-X-R
 R-X-Y-X-R-G-W-I-X-F-X-R
 35 L-X-Y-X-R-G-W-I-X-F-X-V

Snake form-25 R-G-G-R-L-X-Y-X-R-R-R-F-X-V-X-V
 R-G-G-R-R-X-Y-X-R-R-R-F-X-V-X-V
 R-G-G-R-L-X-Y-X-R-R-R-F-X-V-X-R
 5 R-G-G-R-R-X-Y-X-R-R-R-F-X-V-X-R
 R-X-Y-X-R-R-R-F-X-V-X-V
 L-X-Y-X-R-R-R-F-X-V-X-R
 R-X-Y-X-R-R-R-F-X-V-X-R
 L-X-Y-X-R-R-R-F-X-V-X-V

10 and the foregoing snake forms wherein A₇ is W and/or
 A₁₂ is W and/or wherein A₁₄ is W and/or wherein A₁₆ is W
 and/or wherein A₁₇ is G and A₁₈ is R; and/or wherein at
 least one of A₅, A₉, A₁₂ and A₁₆ is not present,
 and the amidated forms thereof,
 15 wherein each X is independently a hydrophobic,
 small, or basic amino acid.

All of these embodiments also include the N-acylated
 and C-amidated forms. X is preferably S, A, T or G, most
 preferably A or T.

20 In all of the foregoing cases, the enantiomeric
 forms, wherein all of the amino acids are in the D-
 configuration are also preferred for use in the methods
 of the invention.

A multiplicity of protegrins have been prepared, and
 25 these include:

PC11: LCYCRRRFCVCVGR
PC12: RCYCRRRFCVCV
PC15: RGGRLCYCRRRFCVCR
PC16: RCYCRRRFCVCR
 30 PC17: LCYCRRRFCVCV
PC18: LCYARRRFAVCV
PC19: RCYARRRFAVCR
PC20: LAYCRRRFCVAV
PC21: RAYCRRRFCVAR
 35 PC22: RGGRLCY RR VCV

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5 PC31: GGRLCYCRRRFCVCV
 PC32: RGRLCYCRRRFCVCV
 PC33: GRLCYCRRRFCVCV
 PC34: RRLCYCRRRFCVCV
 PC35: RLCYCRRRFCVCV
 PC36: RRCYCRRRFCVCV
 PC37: CYCRRRFCVCV
 PC44: RGGRLCYCRRRFCV
 PC47: RGGRLCY RRRF VCV
10 PC48: RGWRLCYCRRRFCVCV
 PC37a: CYCRRRFCVCVGR
 PC45: RGGRLCYCRRRFCV
 PC72: LCYCRRRFCVC
 PC64: LCYTRRRFTVCV
15 PC64a: LTYCRRRFCVTV
 PC31a: GGRLCYCRRRFCVCVGR
 PC32a: RGRLCYCRRRFCVCVGR
 PC33a: GRLCYCRRRFCVCVGR
 PC34a: RRLCYCRRRFCVCVGR
20 PC35a: RLCYCRRRFCVCVGR
 PC36a: RRCYCRRRFCVCVGR
 PC44a: RGGRLCYCRRRFCVCR
 PC47a: RGGRLCY RRRF VCVGR
 PC48a: RGWRLCYCRRRFCVCVGR
25 PC54: RGWRLAYCRRRFCVAVGR
 PC61: RCYCRRRFCVCV
 PC62: LCYCRRRFCVCR
 PC63: VCYCFRRFCYCV
 PC65: LCYTRPRFTVCV
30 PC66: LCYTRGRFTVCV
 PC67: LCYFRRRFIVCV
 PC68: LCYFRPRFIVCV
 PC69: LCYTFRPRFVCV
 PC70: LCYTFRGRFVCV
35 PC74: CYCFRRFCVC

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5 PC77: LCYCRRRRCVCV
 PC78: LCYCFRRRCVCV
 PC79: LCYCRFRRRCVCV
 PC80: LCYCRRFRCVCV
 PC81: LCYCRRFFCVCV
 PC82: LCYCRFFRCVCV
 PC83: LCYCFRRRCVCV
 PC84: LCYCFRRFCVCV
 PC85: LCYCFRFRVCVCV
10 PC86: LCYCRFRFCVCV
 PC87: LCYCFRFFCVCV
 PC88: LCYCFRFRVCVCV
 PC89: LCYCFRRFCVCV
 PC90: LCYCRFFFCVCV
15 RGRLCY RR VCVGR
 PC91: YCYCRRRRCVCVGR
 PC95: ICYCRRRRCVCVGR
 PC96: FCYCRRRRCVCVGR
 PC97: WCYCRRRRCVCVGR
20 PC99: RCYCRRRRCVCVGR
 PC109: RLCYTRGRFTVCV
 PC110: LCYTRGRFTVCVR
 PC111: RLCYTRGRFTVCVR
 PC112: LCYCHHHFCVCV
25 PC113: LCYTHHHFTVCV
 RGGLCYCRRRRCVCVGR
 RGRLCYCRRRRCVCVGR
 RGGGLCYCRRRRCVCVGR
 RGGGLCYCRRGFCVCFGR
30 RGGGLCYCRRPFCVCVGR
 RGGGLCYCRPRFCVCVGR
 RGRLCYCRXRFCVCVGR (X=NMeG)
 RGGLCYCRGRFCVCVGR
 RGRLCYCXGRFCVCVGR (X=Cit)
35 XGGRLCYCRGRFCVCVGR (X=Cit)

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RGGRVCYCRGRFCVCVGR
RGGGLCYCFPKFCVCVGR
RGWGLCYCRPRFCVCVGR
5 RGWRLCYCRXRFCVCVGR (X=NMeG)
RGWRLCYCRGRFCVCVGR
RGWRLCYCXPRFCVCVGR (X=Cit)
RWRLCYCRPRFCVCVGR
RGWRLCYCRPRFCVCVGR
RGWRACYCRPRFCACVGR
10 GWRLCYCRPRFCVCVGR
RWRLCYCKGKFCVCVGR
RGWRLCYCRXRFCVCVGR (X=NMeG)
GGWRLCYCRGRFCVCVGR
RGGWLCYCRGRFCVCVGR
15 RLLRLCYCRXRFCVCVGR (X=NMeG)
RLLRACYCRXRFCVCVGR (X=NMeG)
RLLRLCYCRRRFCVCVGR
RGLRXCYCRGRFCVCVGR (X=Cha)
RGGRLCYCRXRZCVCWGR (X=NMeG) (Z=Cha)
20 RGGRWCVCRXRZCYCVGR (X=NMeG) (Z=Cha)
RGLRXCYCRGRFCVCVGR (X=Cha)
RGGRWCVCRGRXCVCVGR (X=Cha)
RGGRLCYCRRRFCXCVCVGR (X=NMeV)
LCYCRRRFCVCV
25 LCYCRRFCVCV
LCYCRRRFCVCF
LCACRRRACVCV
LCYCRXRFCVCV (X=D-Arg)
LCWCRRRFCVCV
30 WCYCRRRFCVCV
LCYCRRRXCVCV (X=homoPhe)
LCYCRRRXCVCV (X=P-ClPhe)
XCYCRRRFCVCV (X=Cha)
LCYCRRRFCXCVCV (X=DHis)
35 LCYCRRRXCVCV (X=NMeGly)

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LCYCRRRXCV CV (X=NMePhe)
LCYCRRRFCXCV (X=NMeVal)
LCXCRRRXCV CV (X=Cha)
LCGCRRRGCV CV
5 LCACRGRACV CV
RACYCRPRFCACV
RLCYCRPRFCV CF
RLCYCRPRFCV CV
KLCYCKPKFCV CV
10 RLCACRGRACV CV
RLCYCRXRFCV CV (X=NMeGly)
RXCFCRPRFCV CV (X=Cha)
RWCFCRPRFCV CV
WLCYCRRRFCV CV
15 WLCFCRRRFCV CV
FLCFCRRRFCV CV
WLCFCRRRXCV CV (X=NMeF)
WYCYCRRRFCV CV
WXCYCRRRFCV CV (X=Cha)
20 RXCFGRGRZCV CV (X=Cha) (Z=NMeF)
XLCFCRRRZCV CV (X=Cha) (Z=NMeF)
RLCYCRPRFCV CVGR
WLCYCRRRFCV CVGR
WXCYCRRRFCV CVGR (X=Cha)
25 RLCYCRGPF CVCR
RRWCFVCYAGFCYRCR
RRCYCRGRFCG CVGR
RWRCYCGRRFCG CVGR
RARC YCGRRFCG CVGR
30 GWRCYCRGRFCG
RGWACYCRGRFCV
RRCYGRRRFGV CVGR
RGWRLCYGRGRFKV
RGWRLCYCRGRFCV
35 CYCRRRFCV CF

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RGWRLCYCRXRFCVC (X=NMeG)
RGWRGCYCRXRFCGC (X=NMeG)
LCYCRPRFCVCVGR
LCYCKPKFCVCVGR
5 LCYCRGRFCVCVGR
LCYCRPRFCVCVGRGR
RRWCYCRPRFCVCVR
WRLCYCRPRFCVCVGR
GWL CYCRGRFCVCVGR
10 RWLCYCRGRFCVCVGR
RLLCYCRGRFCVCVGR
RWRLCYCRPRFCVCV
RXRLCYCRZRFCVCV (X=Cha) (Z=NMeG)
RGWRLCYCRGRXCVCV (X=Cha)
15 RGGVVCYCRGRFCVCV
LCYCRXRFCVCV (X=D-Ala)
LCYCKPKFCVCV
VCYCRPRFCVCV
LCYCRPRFCVCW
20 LCYRRPRFRVCV
RGWRLCYCRGRXCVCV (X=Cha)
RXRLCYCRZRFCVCV (X=Cha) (Z=NMeG)
RXRLCYCRGRFCVCV (X=Cha)
RGGGLCYARGWIAFCVGR
25 RGGGLCYARGFIAVCFGR
RGGGLCYARPRFAVCVGR
RGGGLCYTRPRFTVCVGR
RGGGLCYARKGFAVCVGR
RGGRLCYARRRFAVCVGR
30 RGGGLCYKRGFIKVCFGR
RGGGLCYKRGWIKFCVGR
RGGGLCYRLPKFRVCVGR
RGGGLCYRLPGFRVCVGR
RGWRGCYKGRFKGCVGR
35 LCYKGRFKVCV

ICYRPRFVCVGR

Preferred such compounds include the free acid and
amidated forms thereof either in linear or disulfide-
5 bridged form, and in the L- or D-enantiomeric forms.

Preparation of the Invention Compounds

The protegrins are essentially peptide backbones
which may be modified at the N- or C-terminus and also
10 may contain one or two disulfide linkages. The peptides
may first be synthesized in noncyclized form. These
peptides may then be converted to the cyclic peptides if
desired by standard methods of disulfide bond formation.
As applied to the protegrins herein, "cyclic forms"
15 refers to those forms which contain cyclic portions by
virtue of the formation of disulfide linkages between
cysteine, homocysteine or penicillamine residues in the
peptide. If the straight-chain forms are preferred, it
is preferable to stabilize the sulfhydryl groups for any
20 peptides of the invention which contain two or more
cysteine, homocysteine or penicillamine residues.

Standard methods of synthesis of peptides the size
of protegrins are known. Most commonly used currently
are solid phase synthesis techniques; indeed, automated
25 equipment for systematically constructing peptide chains
can be purchased. Solution phase synthesis can also be
used and has considerable benefits for large scale
production. When synthesized using these standard
techniques, amino acids not encoded by the gene and
30 D-enantiomers can be employed in the synthesis. Thus,
one very practical way to obtain the compounds of the
invention is to employ these standard chemical synthesis
techniques.

In addition to providing the peptide backbone, the
35 N- and/or C-terminus can be derivatized, again using

conventional chemical techniques. The compounds of the invention may optionally contain an acyl group, preferably an acetyl group at the amino terminus.

Methods for acetylating or, more generally, acylating, the free amino group at the N-terminus are generally known in the art; in addition, the N-terminal amino acid may be supplied in the synthesis in acylated form.

At the carboxy terminus, the carboxyl group may, of course, be present in the form of a salt; in the case of pharmaceutical compositions this will be a pharmaceutically acceptable salt. Suitable salts include those formed with inorganic ions such as NH_4^+ , Na^+ , K^+ , Mg^{++} , Ca^{++} , and the like as well as salts formed with organic cations such as those of caffeine and other highly substituted amines. However, when the compound of formula (1) contains a multiplicity of basic residues, salt formation may be difficult or impossible. The carboxy terminus may also be esterified using alcohols of the formula ROH wherein R is hydrocarbyl (1-6C) as defined above. Similarly, the carboxy terminus may be amidated so as to have the formula $-\text{CONH}_2$, $-\text{CONHR}$, or $-\text{CONR}_2$, wherein each R is independently hydrocarbyl (1-6C) as herein defined. Techniques for esterification and amidation as well as neutralizing in the presence of base to form salts are all standard organic chemical techniques.

If the peptides of the invention are prepared under physiological conditions, the side-chain amino groups of the basic amino acids will be in the form of the relevant acid addition salts.

For synthesis of linear peptide with a C-terminal amide, the peptide sequence is conveniently synthesized on a Fmoc Rink amide solid support resin (Bachem) using Fmoc chemistry on an automated ABI 433 peptide synthesizer (ABD, Perkin Elmer, Foster City, CA)

according to the manufacturer's standard protocols. Cleavage is typically carried out in 10 ml of thioanisole/EDT/TFA (1/1/9) for 2 hours at room temperature. Crude cleavage product is precipitated with
5 t-butyl methyl ether, filtered and dried.

Formation of disulfide linkages, if desired, is conducted in the presence of mild oxidizing agents. Chemical oxidizing agents may be used, or the compounds may simply be exposed to the oxygen of the air to effect
10 these linkages. Various methods are known in the art. Processes useful for disulfide bond formation have been described by Tam, J.P. et al., *Synthesis* (1979) 955-957; Stewart, J.M. et al., Solid Phase Peptide Synthesis, 2d Ed. Pierce Chemical Company Rockford, IL (1984); Ahmed
15 A.K. et al., *J Biol Chem* (1975) 250:8477-8482 and Pennington M.W. et al., *Peptides 1990*, E. Giralt et al., ESCOM Leiden, The Netherlands (1991) 164-166. An additional alternative is described by Kamber, B. et al., *Helv Chim Acta* (1980) 63:899-915. A method conducted on
20 solid supports is described by Albericio *Int J Pept Protein Res* (1985) 26:92-97.

A particularly preferred method is solution oxidation using molecular oxygen. This method has been used by the inventors herein to refold synthetic PG-1,
25 PG-3 in its amide or acid forms, enantio PG-1 and the two unidisulfide PG-1 compounds (C₆-C₁₅ and C₈-C₁₃). Recoveries are as high as 65-90%.

In this preferred method to form disulfide linkages, the crude peptide is dissolved in DMSO and added to 20 mM
30 ammonium acetate buffer, pH 7. The final concentration of the peptide in the solution is between 1-8 µg/mL, the pH ranges from 7.0-7.2, and the DMSO concentration ranges from 5-20%. The peptide solution is stirred overnight at room temperature.

The pH of the solution is adjusted to pH5 with concentrated acetic acid and the sample purified on Prep LC. After loading, the column is washed with 10% acetonitrile/H₂O (0.1% TFA) until the UV absorbance
5 decreases to the baseline. The gradient is then started.

Column: Vydac Cat#218TP101522, 2.2 x 25 cm, C₁₈ peptides & proteins; UV λ : 235 nm; Flow Rate: 10 ml/min.

Solvent A is 100% 0.1% TFA/H₂O; Solvent B is 100% 0.08% TFA/ACN. The gradient is as follows.

10

T (min)	%B (linear gradient)
0	10
10	18
80	32
95	95

Fractions are analyzed by analytical HPLC and those that contain the desired peptide are combined. The acetonitrile is stripped and the resulting aqueous
15 solution lyophilized. The resulting amide, containing sulfide bonds, is confirmed by mass spectrum.

If the peptide backbone is comprised entirely of gene-encoded amino acids, or if some portion of it is so composed, the peptide or the relevant portion may also be
20 synthesized using recombinant DNA techniques. The DNA encoding the peptides of the invention may itself be synthesized using commercially available equipment; codon choice can be integrated into the synthesis depending on the nature of the host.

25 Recombinantly produced forms of the protegrins may require subsequent derivatization to modify the N- and/or C-terminus and, depending on the isolation procedure, to effect the formation of disulfide bonds as described hereinabove. Depending on the host organism used for
30 recombinant production and the animal source from which

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the protein is isolated, some or all of these conversions may already have been effected.

For recombinant production, the DNA encoding the protegrins of the invention is included in an expression system which places these coding sequences under control of a suitable promoter and other control sequences compatible with an intended host cell. Types of host cells available span almost the entire range of the plant and animal kingdoms. Thus, the protegrins of the invention could be produced in bacteria or yeast (to the extent that they can be produced in a nontoxic or refractile form or utilize resistant strains) as well as in animal cells, insect cells and plant cells. Indeed, modified plant cells can be used to regenerate plants containing the relevant expression systems so that the resulting transgenic plant is capable of self protection vis-à-vis these infective agents.

The protegrins can be produced in a form that will result in their secretion from the host cell by fusing to the DNA encoding the protegrin, a DNA encoding a suitable signal peptide, or may be produced intracellularly. They may also be produced as fusion proteins with additional amino acid sequence which may or may not need to be subsequently removed prior to the use of these compounds as antimicrobials or antivirals.

Thus, the protegrins can be produced in a variety of modalities including chemical synthesis and recombinant production or some combination of these techniques.

30 Compositions Containing the Protegrins and Methods of Use

The protegrins of the invention are effective in inactivating Gram-negative bacteria that are the cause of periodontal disease.

For use as a treatment of periodontal conditions, the protegrins of the invention can be formulated as

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pharmaceutical or veterinary compositions. Depending on the subject to be treated, the mode of administration, and the type of treatment desired -- e.g., prevention, prophylaxis, therapy; the protegrins are formulated in
5 ways consonant with these parameters. A summary of such techniques is found in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Co., Easton, PA.

The protegrins can be used in animal subjects both
10 as therapeutic and prophylactic treatments; by "treating" an infection is meant either preventing it from occurring, ameliorating the symptoms, inhibiting the growth of the microbe in the subject, and any other negative effect on the microbe which is beneficial to the subject. Thus,
15 "treating" or "treatment" have both prophylactic and therapeutic aspects.

The protegrins are particularly attractive as an active ingredient in pharmaceutical compositions useful in treatment of periodontal diseases. Topical
20 formulations are preferred and include creams, salves, oils, powders, gels and the like. Suitable topical excipients are well known in the art and can be adapted for particular uses by those of ordinary skill.

In general, for use in therapy or prophylaxis of
25 periodontal disease, the protegrins of the invention may be used alone or in combination with other antibiotics such as erythromycin, tetracycline, macrolides, for example azithromycin and the cephalosporins. Depending on the mode of administration, the protegrins will be
30 formulated into suitable compositions to permit facile delivery to the affected areas. The protegrins may be used in forms containing one or two disulfide bridges or may be in linear form. In addition, use of the enantiomeric forms containing all D-amino acids may
35 confer advantages such as resistance to those proteases,

such as trypsin and chymotrypsin, to which the protegrins containing L-amino acids are less resistant. Of course, mixtures of protegrins can be used.

The protegrins can be administered singly or as
5 mixtures of several protegrins or in combination with other pharmaceutically active components. The formulations may be prepared in a manner suitable for systemic administration or topical or local
10 administration. Systemic formulations include those designed for injection (e.g., intramuscular, intravenous, intraperitoneal or subcutaneous injection) or may be prepared for transdermal, transmucosal, or oral
administration. The formulation will generally include a diluent as well as, in some cases, adjuvants, buffers,
15 preservatives and the like. The protegrins can be administered also in liposomal compositions or as microemulsions.

If systemic administration is to be oral, the protegrins of the invention should be protected from
20 degradation in the digestive tract using a suitable enteric coating. This may be avoided to some extent by utilizing amino acids in the D-configuration, thus providing resistance to protease. The protegrins are relatively acid stable, however, some degree of enteric
25 coating may still be required.

For use in treating periodontal disease, of course, it is preferred to use topical compositions that can be applied directly in the mouth. It is significant that the protegrins are effective antimicrobials in the
30 presence of saliva. The compositions can be applied directly to the affected areas using standard techniques known in the art.

The following examples illustrate but do not limit the invention:

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Preparation APreparation Of Test Bacteria

Bacteria, including *A. actinomycetemcomitans* ATCC 29523, FDC-Y4, NCTC 9709, *Capnocytophaga sputigena* ATCC 33123, *Capnocytophaga gingivalis* ATCC 33124, and *Capnocytophaga ochracea* ATCC 27872 were grown on Laked blood agar overnight, and suspended in trypticase soy broth (BBL Microbiology; Cockeysville, Md.) containing 0.1% sodium bicarbonate, 0.05% equine hemin III (Sigma Chemical Co.; St. Louis, Mo.), 0.0001% menadione (Sigma) and 0.1% yeast extract (Difco Laboratories, Detroit, Mich.). The bacteria were incubated an additional 4 h to early log growth phase.

15

Preparation BPreparation of Protegrins

Protegrin 1 (PG-1), an enantiomer of protegrin 1 comprised of all D-amino acids (D-PG-1), protegrin 2 (PG-2), protegrin 3 (PG-3), and protegrin 5 (PG-5) were synthesized using Fmoc chemistry (SynPep; Dublin, CA). The crude, synthetic peptides were reduced with dithiothreitol and purified by reversed phase HPLC on a Vydac C₁₈ silica column (1 x 25 cm; The Separations Group; Hesperia, CA) using an acetonitrile gradient in the presence of 0.1% aqueous trifluoroacetic acid, and concentrated by vacuum centrifugation (Speed-Vac; Savant Instruments, Farmington, NY). The reduced peptides (0.1-0.2 µg/mL) were subjected to air oxidation, 24-48 h, in 0.1 mol/L tris, pH 7.7, to allow the formation of intramolecular cystine disulfide bonds. The peptides were purified by reversed phase HPLC using the Vydac C₁₈ silica column. The peptide preparations used in this study were homogenous as assessed by reversed phase HPLC, acid-urea polyacrylamide gel electrophoresis, and fast atom bombardment-mass spectrometry. Stock solutions were

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prepared in glass distilled water. Concentrations of PG-1 were verified upon an extinction coefficient of $1.280 \text{ (mmol/L)}^{-1} \text{ cm}^{-1}$ at 280 nm.

Example 1Sensitivity of Periodontal Bacteria to the Protegrins

In all of the assays conducted, bacterial concentrations were adjusted turbidometrically such that the final concentration in the bactericidal assay was approximately 10^7 cells/mL in Hank's balanced salt solution (HBSS; Sigma Chemical Co., St. Louis MO), pH 7.0. The bacterial suspension, protegrin, and any other additive (such as serum) were admixed in a final volume of 40 μ L. The mixture was incubated at 37°C in a temperature block for the time periods specified in the results. The reaction was terminated by dilution, 1:100 in HBSS, and plating using a Spiral plater, Model D (Spiral Biotech, Inc.; Bethesda, MD). Colony-forming units (CFU) were enumerated after 48-72 h incubation. Bactericidal activity was expressed as the \log_{10} reduction in CFU ($\delta\log_{10}$). The 99% effective dose (ED_{99}) is the theoretical concentration of protegrin peptide at which the $\delta\log_{10}$ is 2.

The effect of PG-1 on various strains of *Actinobacillus actinomycetemcomitans* and *Capnocytophaga* Spp. are shown in Table 1.

Table 1

Organism	Strain	$ED_{99}, \mu\text{g/mL}^a$	n
<i>A. actinomycetemcomitans</i>	ATCC 29523	1.3 ± 1.0	5
<i>A. actinomycetemcomitans</i>	FDC-Y4	0.9 ± 0.7	5
<i>A. actinomycetemcomitans</i>	NCTC 9709	0.7 ± 0.2	5
<i>C. sputigena</i>	ATCC 33123	14.6 ± 11.1	5
<i>C. gingivalis</i>	ATCC 3124	6.2 ± 4.2	5
<i>C. ochracea</i>	ATCC 27872	21.2 ± 10.7	5

^a Incubated 1 h, 37°C. Means \pm standard deviations from values interpolated from dose-response curves

The results are shown as ED₉₉, 1 hour-i.e., 99% effective doses. In general, strains of *A. actinomycetemcomitans* were an order of magnitude more sensitive than those of *Capnocytophaga Spp*. Kinetic analysis showed exponential killing although there was occasionally a brief lag phase. An alternative presentation of these results is shown in Figure 2.

In addition to PG-1, its D-enantiomer and various synthetic protegrins including PG-2, PG-3 and PG-5 were tested, with the results set forth in Figure 3. Figure 3A represents killing of *A. actinomycetemcomitans* ATCC 29523; panel 3B shows killing of another strain of this organism, FDC-Y4; and panel 3C shows killing of the NCTC-9709 strain. Panels 3D, 3E and 3F show killing of *C. sputigena* ATCC 33123; *C. gingivalis* ATCC 33124, and *C. ochracea* ATCC 27872 respectively.

Bacterial activity is shown as δLog_{10} (please explain) and the bars represent the mean and standard deviation of three trials. The clear bar represents no additions; and, subsequently reading left to right the bars represent 100 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, 1 $\mu\text{g/mL}$ and 0.1 $\mu\text{g/mL}$ of the protegrin respectively.

It is seen that all of these protegrins were affective against *A. actinomycetemcomitans* between 1-10 $\mu\text{g/mL}$ and against strains of *Capnocytophaga Spp* between 10-100 $\mu\text{g/mL}$. While all strains of *A. actinomycetemcomitans* were comparably affected by all congeners, for the strains of *Capnocytophaga*, the profile of sensitivities was D-PG-1=PG-5 > PG-2 = PG-1 \geq equal to PG-3.

Example 2

Effect of Serum and Tonicity

The method of Example 1 was repeated in the presence of 20% normal human serum. The results are shown in Figure 4A, 4B and 4C. Human serum antagonizes the bactericidal effects of PG-1 against both serum sensitive
5 *A. actinomycetemcomitans* and serum sensitive *Capnocytophaga*. This was not clearly demonstrable against *Capnocytophaga* Spp. unless the serum was first inactivated by heat. Serum, 20% (v/v), usually did not block the bactericidal effects of PG-1 at concentrations
10 of PG-1 equal to or greater than 100 µg/mL. When the concentration of PG-1 was reduced to 10 µg/mL, no killing was observed above 10% (v/v) serum (Fig. 4C).

Figure 4C shows that serum at 10% (v/v) or less does not inhibit bactericidal activity of 10 µg/mL PG-1. The
15 protegrins were bactericidal in HBSS, thus the protegrins are relatively insensitive to tonicity. The concentrations of NaCl, KCl, and NaBr in 10 mmol/L sodium phosphate buffer, pH 7.2 were varied; the protegrins lost their bactericidal activity only under hypertonic
20 conditions, above 0.5 mol/L NaCl, KCl, and NaBr (Fig. 5).

Claims

1. A method to treat periodontal disease which
5 method comprises administering to a subject afflicted
with such disease an amount of a protegrin effective to
treat said disease; wherein said protegrin contains the
amino acid sequence:

10 $A_1-A_2-A_3-A_4-A_5-C_6-A_7-C_8-A_9-A_{10}-A_{11}-A_{12}-C_{13}-A_{14}-C_{15}-A_{16}-A_{17}-A_{18}$ (1)

wherein said protegrin contains 10-30 amino acid
residues, wherein the amino acid sequence of formula (1)
may be extended at the N and/or C-terminus by additional
15 noninterfering amino acids;

and the N-terminal acylated and/or C-terminal
amidated or esterified forms thereof, said protegrin
either in the optionally -SH stabilized linear or in a
disulfide-bridged form

20 wherein each of C_6 , C_8 , C_{13} and C_{15} is independently
a cysteine, homocysteine, or penicillamine, or wherein
one or more of C_6 , C_8 , C_{13} and C_{15} is independently
replaced by a basic, hydrophobic, large/polar or small
amino acid or wherein C_8 and/or C_{13} is not present;

25 each of A_1-A_5 is independently present or not
present, and if present each is independently a basic,
hydrophobic, polar/large, or small amino acid;

each of A_7 and A_{14} is independently a hydrophobic or
a small amino acid;

30 A_9-A_{12} are capable of effecting a β -turn when
contained in the compound of formula (1) and at least one
of A_9-A_{12} must be a basic amino acid and wherein A_9 and/or
 A_{12} may be present or not present;

each of A₁₆-A₁₈ is independently present or not present, and if each present each is independently a basic, hydrophobic, polar/large or small amino acid;

wherein in said protegrin at least about 15% to
5 about 50% of the amino acids are basic amino acids, and wherein the protegrin compound has a net positive charge of at least +1 at physiological pH.

2. The method of claim 1 wherein said protegrin
10 contains two disulfide bridges.

3. The method of claim 1 wherein said protegrin contains one disulfide bridge.

15 4. The method of claim 1 wherein said protegrin is in the linear form.

5. The method of any of claims 1-4 wherein A₇ and A₁₄ are hydrophobic, and/or
20 wherein A₅ and A₁₆ are hydrophobic or small; and/or wherein at least one of A₉ and A₁₂ is hydrophobic; and/or
wherein A₁₀ and A₁₁ is each independently proline, or small or a basic or hydrophobic amino acid; and/or
25 wherein C₈ and C₁₃ are independently cysteine, homocysteine or penicillamine and the amino acids at A₉ and A₁₂ are present; and/or
wherein at least one of A₁-A₅ is not present; and/or wherein at least one of A₁-A₄ is hydrophobic.

30

6. The method of claim 5 wherein each of A₅ and A₁₆ is independently selected from the group consisting of I, V, L, NLe, W, Y and F; and/or

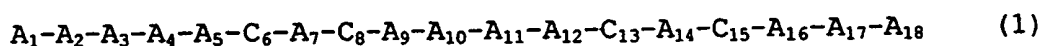
wherein each of A₇ and A₁₄ is independently selected
35 from the group consisting of I, V, L, W, Y and F; and/or

wherein said protegrin is in the D-enantiomeric form.

7. The method of claim 6 wherein said protegrin is
5 PG-1, PG-2, PG-3, PG-4 or PG-5 or the enantiomeric form thereof.

8. A pharmaceutical composition for treatment of periodontal disease use which comprises the protegrin of
10 any of claims 1-7 in admixture with at least one pharmaceutically acceptable excipient.

9. Use of a protegrin to prepare a medicament to treat periodontal disease wherein said protegrin
15 contains the amino acid sequence:



wherein said protegrin contains 10-30 amino acid
20 residues, wherein the amino acid sequence of formula (1) may be extended at the N and/or C-terminus by additional noninterfering amino acids;

and the N-terminal acylated and/or C-terminal amidated or esterified forms thereof, said protegrin
25 either in the optionally -SH stabilized linear or in a disulfide-bridged form

wherein each of C_6 , C_8 , C_{13} and C_{15} is independently a cysteine, homocysteine, or penicillamine, or wherein
one or more of C_6 , C_8 , C_{13} and C_{15} is independently
30 replaced by a basic, hydrophobic, large/polar or small amino acid or wherein C_8 and/or C_{13} is not present;

each of A_1-A_5 is independently present or not present, and if present each is independently a basic, hydrophobic, polar/large, or small amino acid;

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each of A₇ and A₁₄ is independently a hydrophobic or a small amino acid;

A₉-A₁₂ are capable of effecting a β -turn when contained in the compound of formula (1) and at least one of A₉-A₁₂ must be a basic amino acid and wherein A₉ and/or A₁₂ may be present or not present;

each of A₁₆-A₁₈ is independently present or not present, and if each present each is independently a basic, hydrophobic, polar/large or small amino acid;

wherein in said protegrin at least about 15% to about 50% of the amino acids are basic amino acids, and wherein the protegrin compound has a net positive charge of at least +1 at physiological pH.

10. The use of claim 9 wherein said protegrin contains two disulfide bridges.

11. The use of claim 9 wherein said protegrin contains one disulfide bridge.

12. The use of claim 9 wherein said protegrin is in the linear form.

13. The use of claim 9 wherein A₇ and A₁₄ are hydrophobic, and/or

wherein A₅ and A₁₆ are hydrophobic or small; and/or

wherein at least one of A₉ and A₁₂ is hydrophobic; and/or

wherein A₁₀ and A₁₁ is each independently proline, or small or a basic or hydrophobic amino acid; and/or

wherein C₈ and C₁₃ are independently cysteine, homocysteine or penicillamine and the amino acids at A₉ and A₁₂ are present; and/or

wherein at least one of A₁-A₅ is not present; and/or

wherein at least one of A₁-A₄ is hydrophobic.

14. The use of claim 9 wherein each of A₅ and A₁₆ is independently selected from the group consisting of I, V, L, NLe, W, Y and F; and/or

5 wherein each of A₇ and A₁₄ is independently selected from the group consisting of I, V, L, W, Y and F; and/or wherein said protegrin is in the D-enantiomeric form.

10 15. The use of claim 9 wherein said protegrin is PG-1, PG-2, PG-3, PG-4 or PG-5 or the enantiomeric form thereof.

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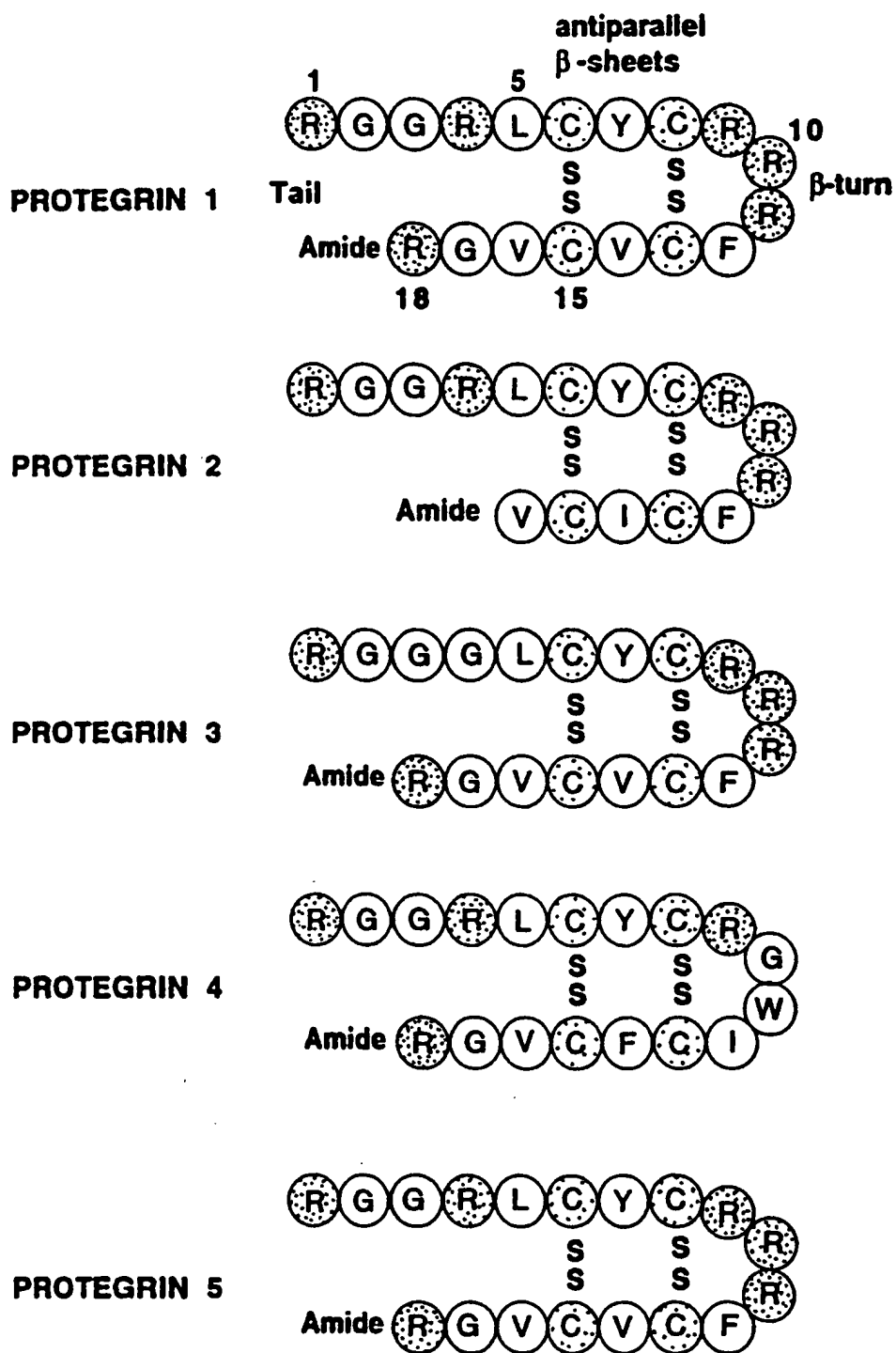


FIG. 1

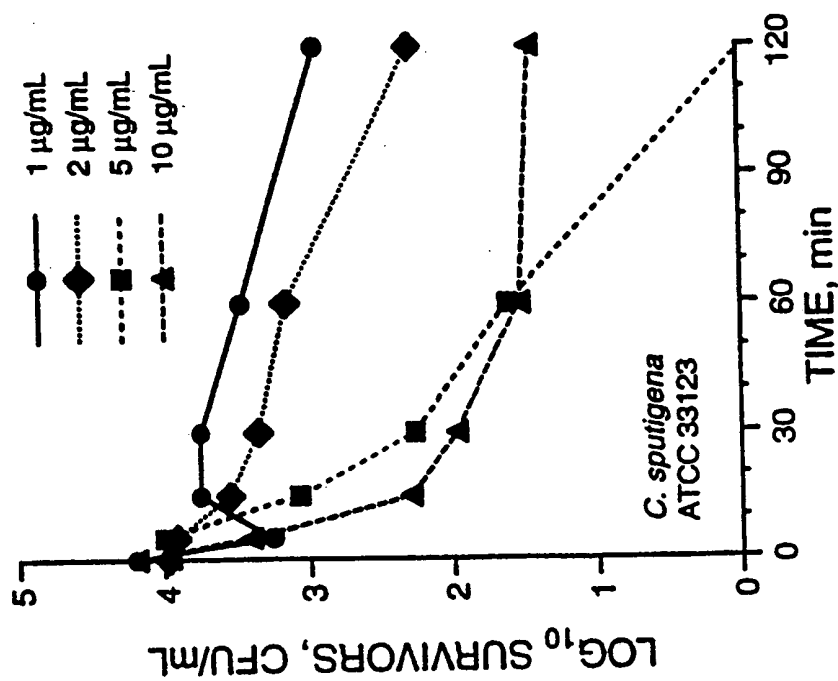


FIG. 2B

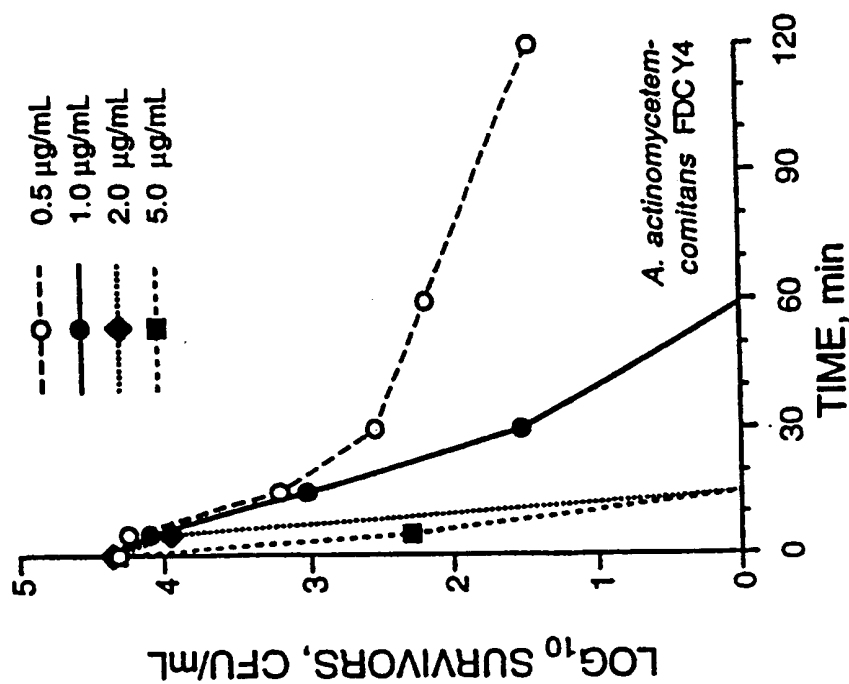


FIG. 2A

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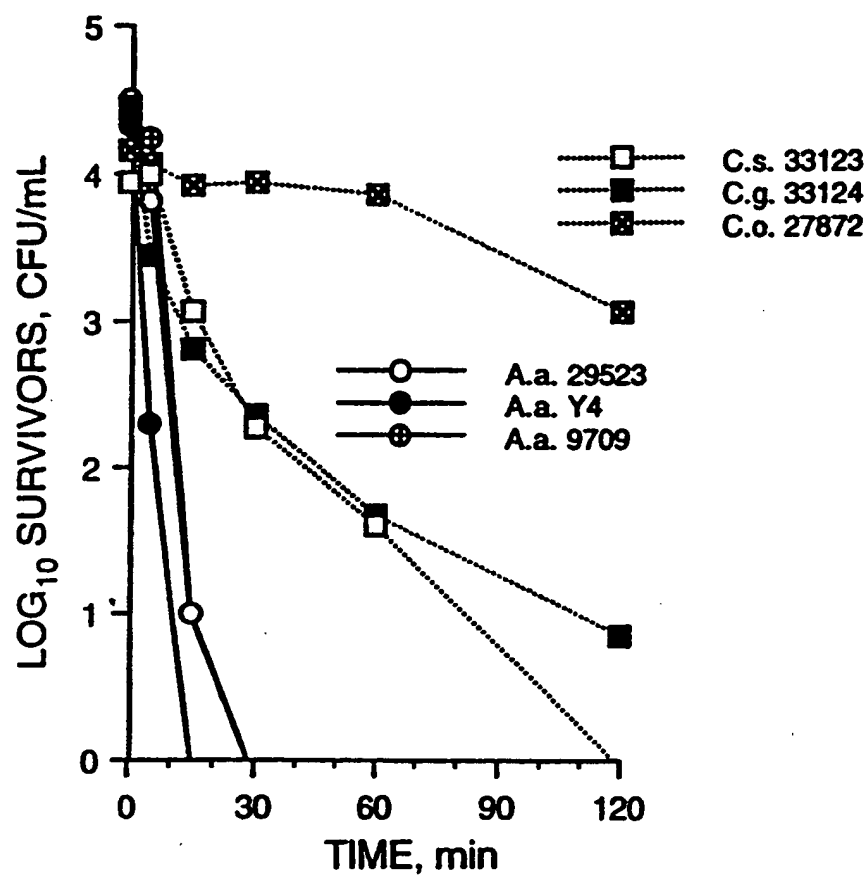


FIG. 2C

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BACTERICIDAL ACTIVITY,

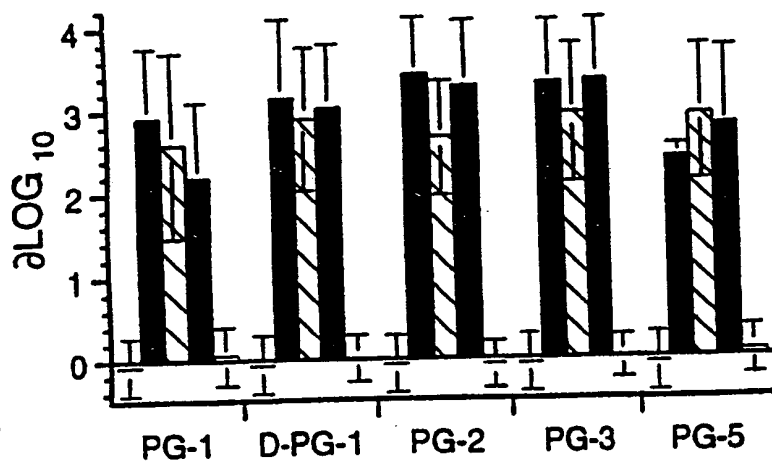


FIG. 3A

BACTERICIDAL ACTIVITY,

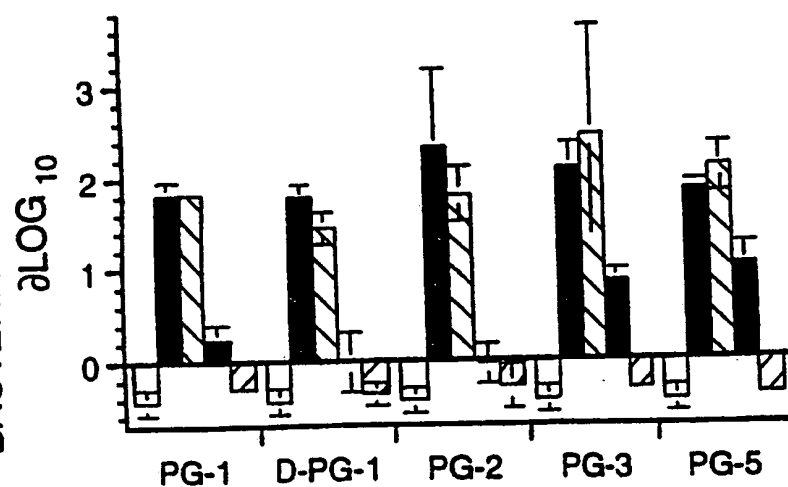


FIG. 3B

BACTERICIDAL ACTIVITY,

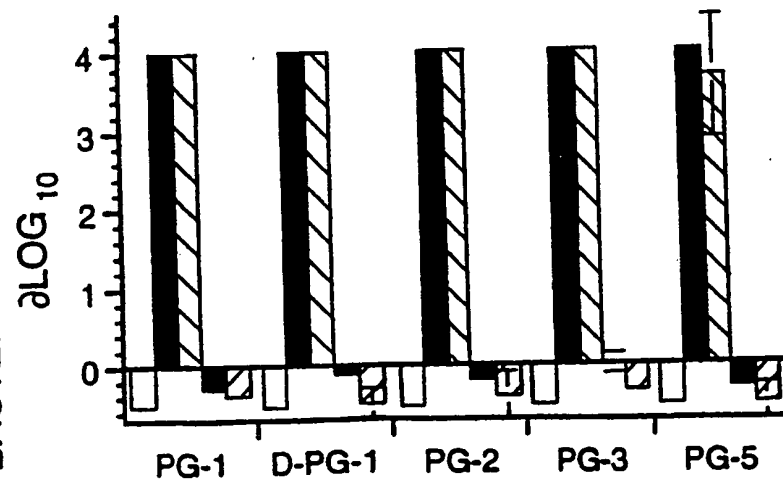


FIG. 3C

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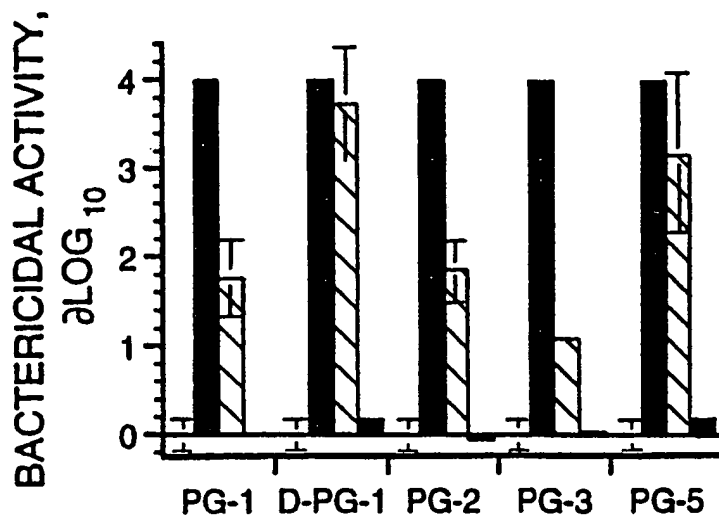


FIG. 3D

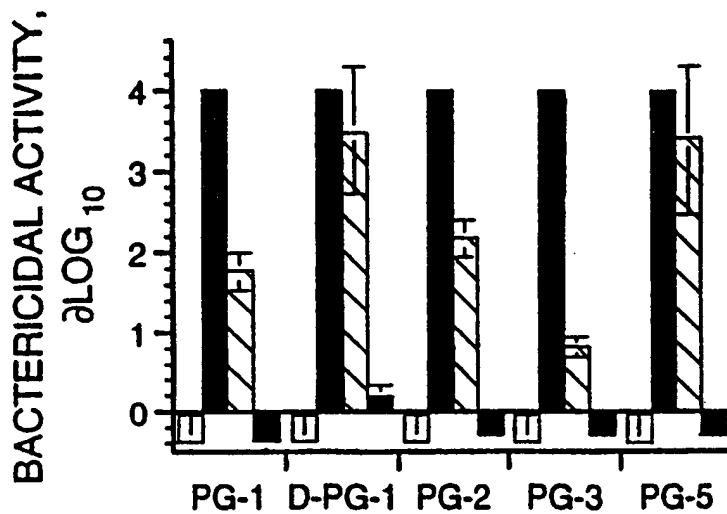


FIG. 3E

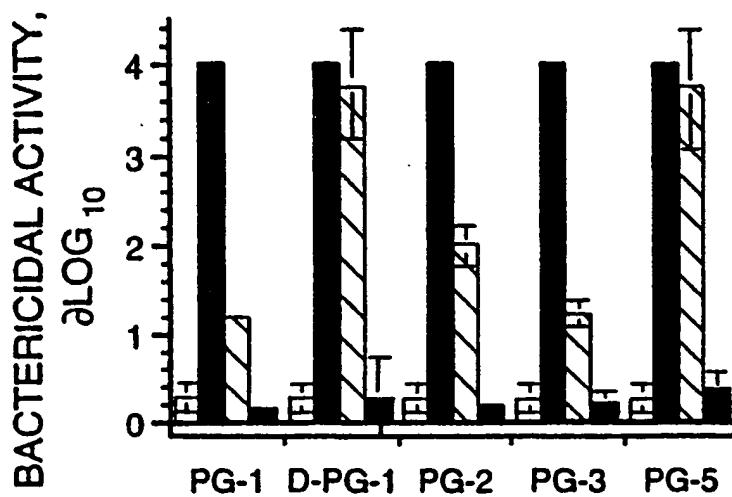


FIG. 3F

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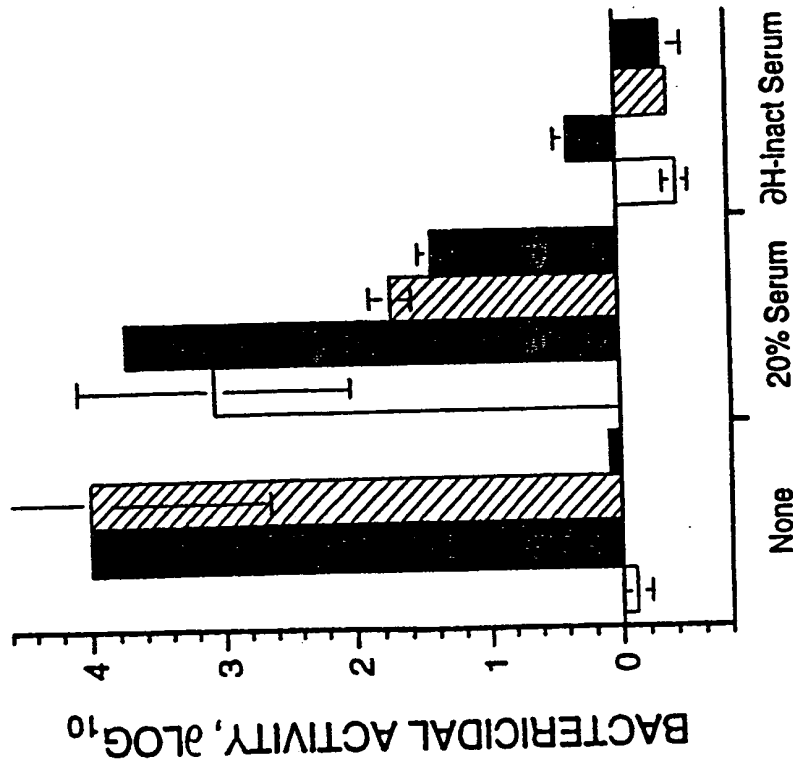


FIG. 4B

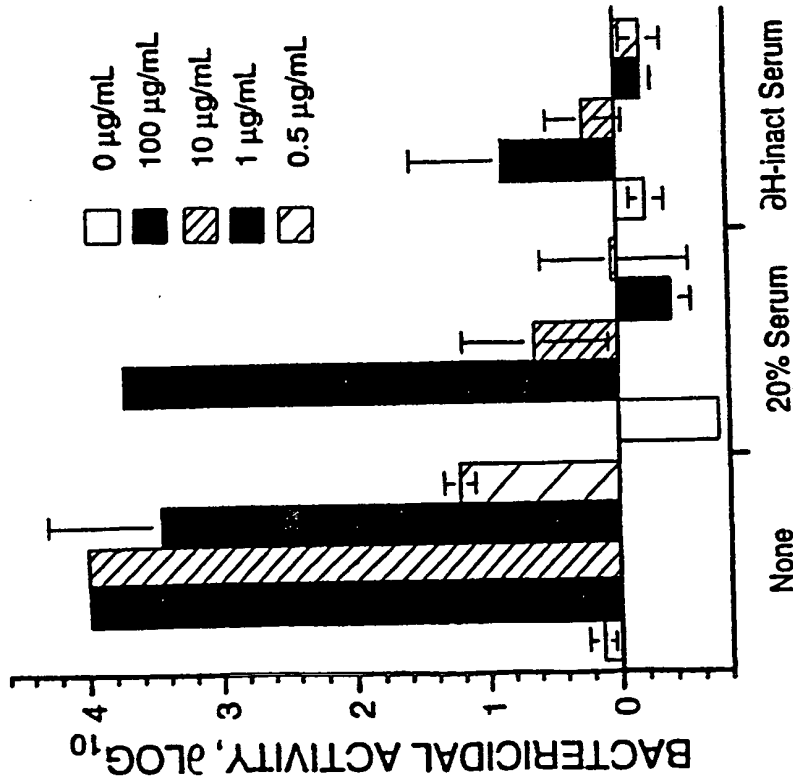


FIG. 4A

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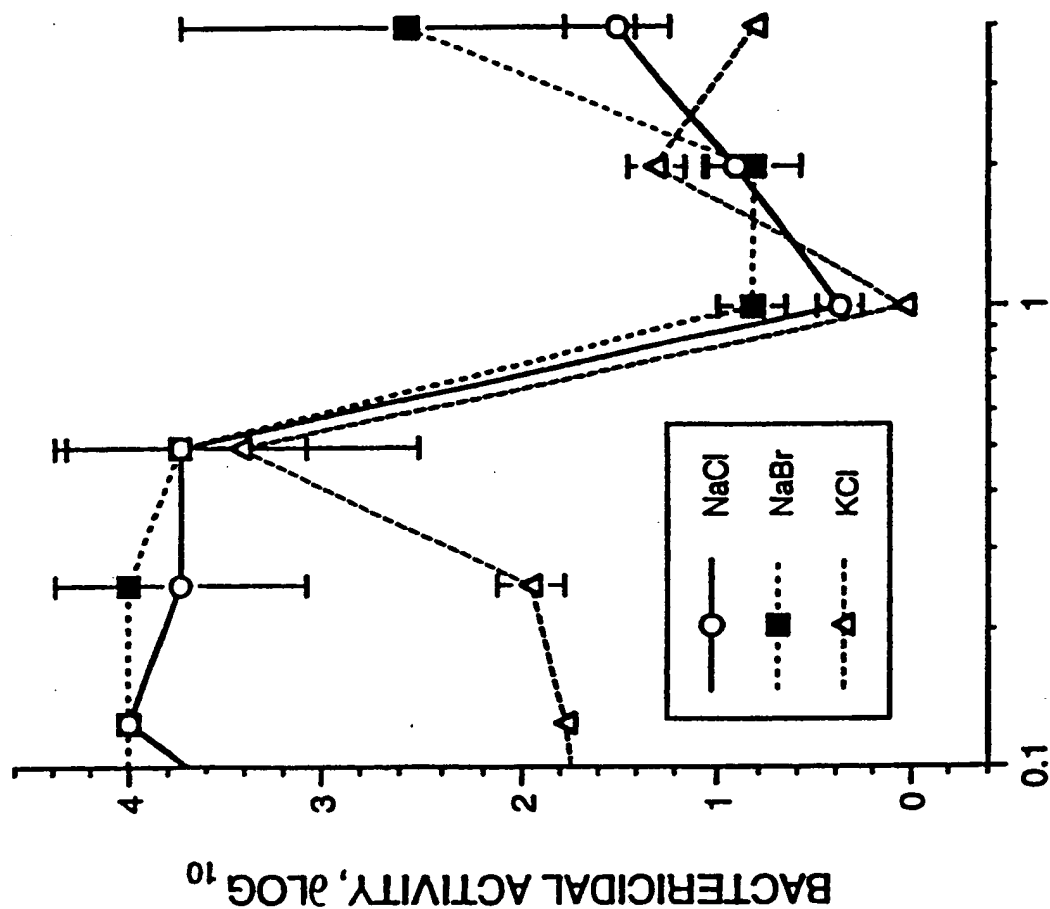


FIG. 5

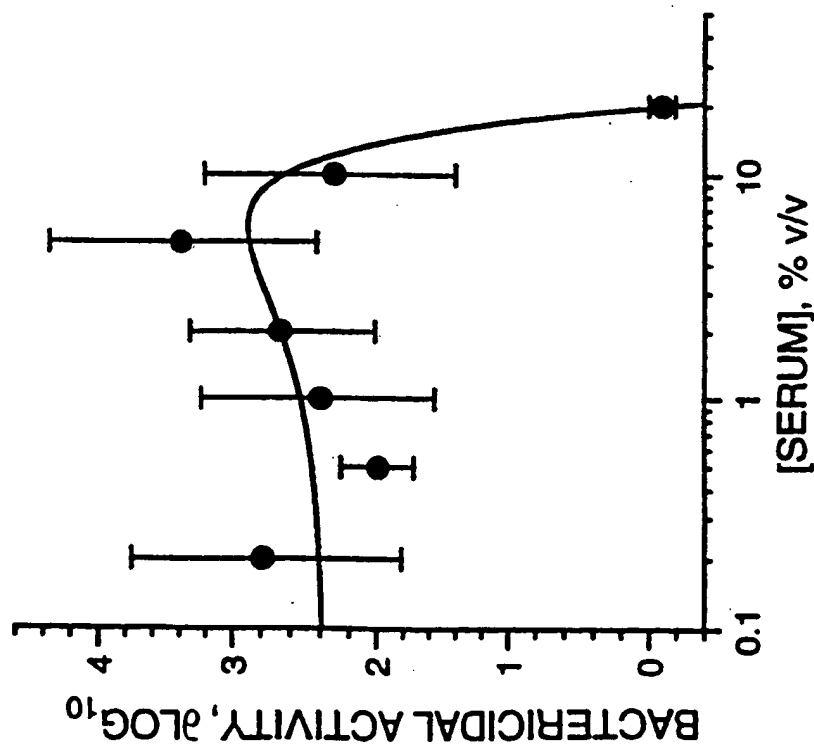


FIG. 4C

INTERNATIONAL SEARCH REPORT

National Application No
PCT/US 98/05362

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/10 C07K7/08 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 37508 A (UNIV CALIFORNIA LOS ANGELES) 28 November 1996 see claims	1-15
Y	WO 95 03325 A (UNIV CALIFORNIA LOS ANGELES) 2 February 1995 see claims	1-15
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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22 July 1998

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

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